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Genetic and Biochemical Analysis of the Bryan Strain of Rous Sarcoma Virus

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Genetic and Biochemical Analysis of the Bryan Strain of Rous Sarcoma Virus

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by

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Dedication

To my daughter Bat-Sheva, may she grow wise enough to understand this.

SUMMARY

Integration of Rous sarcoma virus DNA into its host genome was analyzed under conditions where secondary integration via virus spread was inhibited. This was accomplished by using the noninfectious *pol*$^-$,*env*$^-$ alpha variant of the Bryan high titer strain of Rous sarcoma virus (BH-RSV). Twelve independent BH-RSV-transformed chicken embryo fibroblast clones were obtained and the provirus-cell junction fragments were mapped by restriction endonuclease cleavage and Southern blotting analyses. We found that expression of the viral genes could occur after proviral integration at many sites on the chicken genome and that there was no apparent preference for specific integration sites.

BH-RSV DNA was further analyzed in order to precisely determine the defects in this strain. A 5 kb *Eco*RI fragment which contained the entire *pol* and *src* genes was molecularly cloned from integrated proviruses of BH-RSV alpha and its *pol*$^+$ parent BH-RSV beta.

DNA sequencing of the *pol*-*src* junction of BH-RSV revealed that the *env* sequence was almost entirely absent; only 6 bp following the *pol* stop codon remained. Starting at position 7 (relative to the end of *pol*), a 91 bp sequence identical to the 91 bp immediately upstream from *src* in other strains of Rous sarcoma virus (RSV) was found. This was followed by the *src* coding sequence. It appears remarkable that in all RSV strains, including this defective BH-RSV, as well as in

cellular-*src* DNA, these 91 bp are conserved.

The helper virus-related sequence of about 100 bp, which is present as a direct repeat in the 5' and 3' sides of *src* in other RSVs, was present only on the 3' side of *src* in BH-RSV. The sequence of about 100 nucleotides immediately following *src* in BH-, PR-, and SR-RSV seems to be completely unconserved.

Restriction enzyme mapping analysis showed the structure of the BH-RSV alpha *pol* gene to be basically unchanged from that of BH-RSV beta. Using cloned DNAs, we have constructeds molecular clones in which the *pol* gene of non-defective RSV was replaced with the *pol* gene of BH-RSV alpha or BH-RSV beta. The BH-RSV alpha *pol* gene was found to be biologically inactive in a transfection assay. Further in vitro recombination localized the lesion to an 859 bp *Xba*I-*Bgl*II fragment in the second third of the *pol* gene.

Analysis of the proteins synthesized in BH-RSV alpha-infected cells revealed the fact that BH-RSV alpha directed the synthesis of a full-sized Pr180 *gag*-*pol* precursor, yet no polymerase related proteins were found in BH-RSV alpha virion particles. The *pol* lesion in the BH-RSV alpha genome appears to cause a defect in the processing or packaging of reverse transcriptase.

LIST OF ABBREVIATIONS

ALV	:	avian leukosis virus
AMV	:	avian myeloblastosis virus
ATP	:	adenosine triphosphate
BH-RSV	:	Bryan high titer strain of Rous sarcoma virus
bp	:	base pairs
CEF	:	chicken embryo fibroblasts
CTP	:	cytosine triphosphate
cDNA	:	complementary DNA
chf	:	chicken helper factor
DTT	:	dithiothreitol
EDTA	:	ethylene diamine tetracetic acid
ev	:	endogenous virus
FSV	:	Fujinami sarcoma virus
gs	:	group specific antigen
kb	:	kilobases
kD	:	kilodalton
LTR	:	long terminal repeat
moi	:	multiplicity of infection
MuLV	:	murine leukemia virus
PR-RSV	:	Prague strain of Rous sarcoma virus
RAV	:	Rous associated virus
RSV	:	Rous sarcoma virus
SDS	:	sodium dodecyl sulfate
SR-RSV	:	Schmidt-Ruppin strain of Rous sarcoma virus
td	:	transformation defective
ts	:	temperature sensitive
Y73	:	Yamaguichi 73 sarcoma virus

A Note on Nomenclature: This thesis presents studies on two isolates of BH-RSV: BH-RSV alpha (genotype *gag*$^+$, *pol*$^-$, *env*$^-$, *src*$^+$) and BH-RSV beta (genotype *gag*$^+$, *pol*$^+$, *env*$^-$, *src*$^+$). In the text, the term "BH-RSV" was used in a general sense to refer to both BH-RSV alpha and beta. The terms "BH-RSV alpha" and "BH-RSV beta" were used to refer to a specific isolate.

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INTRODUCTION

Classification. Avian RNA tumor viruses are classified in the family Retrovirdae, subfamily Oncovirinae [91]. Retroviridae, or retroviruses, are positive strand RNA viruses which contain an RNA-dependent DNA polymerase (reverse transcriptase, E.C. 2.7.7.7) and replicate via a DNA intermediate. Avian RNA tumor viruses (also known as avian sarcoma and leukemia viruses) can be further classified into two groups, slowly transforming viruses and rapidly transforming viruses, on the basis of both pathology and genetics.

Slowly transforming viruses are replication competent viruses that may cause neoplastic disease in vivo after a relatively long latent period and generally do not affect the morphology of cells in tissue culture. These viruses do not contain genes other than those needed for replication. Rapidly transforming viruses are usually defective for replication but cause rapid neoplastic disease in vivo and morphological transformation of cells in tissue culture. Rapidly transforming viruses contain a specific gene which is responsible for their oncogenic and transforming abilities. The tranforming genes, generically known as onc genes, are highly related to normal cellular genes (c-onc genes). Rapidly transforming viruses are believed to have arisen via recombination events between slowly transforming viruses and c-onc genes.

Virus Particle. Retroviruses possess an enveloped particle which is released from host cells by the process of budding.

The physical particle (virion) of avian retroviruses consists of: i) an internal core of viral structural proteins, p19, p10, p27, p12, and p15, ii) an outer membrane derived from the host plasma membrane, and iii) viral surface glycoproteins embedded within the outer membrane, gp85 and gp37. Two chemically and genetically identical subunits of RNA and 10-20 molecules of reverse transcriptase are packaged within the viral core. Each RNA subunit is a complete viral genome, 8-10 kb (34-39S sedimentation value) in non-defective viruses. The RNA is single stranded, capped at its 5' end, and polyadenylated on its 3' end. The two genomic RNA molecules are hydrogen bonded at or near their 5' ends, forming a 60-70S viral RNA complex. Also within the core, is a selected population of cellular tRNAs and low molecular weight ribosomal RNAs, including $tRNA^{trp}$, the primer for negative strand viral DNA synthesis.

Life Cycle. Virus can be adsorbed non-specifically to the host's cell surface; however, penetration requires a specific interaction between the viral glycoprotein and receptors on the host cell membrane. Penetration will not occur if the virus lacks surface glycoproteins or if the cells lack the proper receptors. Once inside the cell, the virus uncoats by an unknown mechanism and viral RNA is reverse transcribed in the cytoplasm. Double stranded viral DNA is transported to the nucleus and integrates into the host cell DNA. Integrated viral DNA (provirus) then behaves like a stable Mendelian gene.

The provirus serves as the template for viral RNA synthesis. Transcription of viral RNA is controlled by a viral

promoter mapped to the U_3 region [66,152], which is located on the 5' end of the provirus (see below). Host nucleic acid and protein synthesis are not turned off as a result of the activity of the virus; the provirus is essentialy a subset of the host's actively transcribed genes. Viral mRNAs consist of both genomic-sized RNA and sub-genomic sized RNA species, the latter generated by splicing.

Viral proteins are assembled at the cell membrane. Virion cores containing reverse transcriptase and viral RNA (indistinguishable from genomic-sized mRNA) bud through the cell membrane into the extracellular medium. The host cell is usually not killed as a result of viral infection, and once infected, the cell continues to release viral particles throughout its lifespan. Viral genetic information is thus transmitted both by infection of new cells (horizontal transmission) and by cell division of infected cells (vertical transmission).

Reverse Transcription and Integration. Viral DNA differs from its RNA template in that it contains a long terminal repeated sequence (LTR) at both ends. LTRs are composed of sequences which derive from 1) the 3' end of viral RNA (U_3), 2) from a short direct repeat found at both ends of viral RNA (R), and 3) from the 5' end of viral RNA (U_5). The order of these sequences is U_3-R-U_5 and, in the avian system, the approximate size of these regions is 250, 20, and 80 bp respectively. Generation of a terminal repeat derived from both ends of viral RNA implies that the process of reverse transcription is more complex than merely copying a viral template.

Reverse transcriptase requires a primer to initiate DNA synthesis. In avian retroviruses this is supplied as a molecule of $tRNA^{trp}$ which is base-paired at its 3' terminus to a stretch of viral RNA (16 bp) located immediately following U_5. Synthesis of DNA complementary to viral RNA (minus strand DNA) utilizes the 3' OH of the tRNA as a primer and proceeds in a 5' to 3' direction (i.e., opposite direction with respect to viral RNA) for 101 bp ("minus strong stop DNA"), until the 5' terminus of viral RNA is reached. The nascent DNA transcript then "jumps" and hybridizes to an R sequence on the 3' end of another viral RNA molecule. Synthesis of minus strand DNA continues in a continuous fashion up to the primer binding site. The minus strand DNA is thus a complementary copy of VIRAL GENOME-U_3-R-U_5.

The primer for plus strand synthesis has not been identified, but is presumed to bind to a highly conserved polypurine tract (PPT) which immediately precedes U_3. Plus strand synthesis appears to initiate before completion of the minus strand. Initiation at the proposed primer binding site and synthesis in the 5' to 3' direction (i.e., same direction as viral RNA) would generate a DNA transcript of U_3-R-U_5 ("plus strong stop DNA"). Continued reverse transcription of the 3' end of $tRNA^{trp}$ would generate a region complementary to the 3' end of minus strand DNA (5' end of genomic RNA) and would facilitate a second jump to a new template molecule or circularization of the growing minus and plus strands. The minus strand acquires its second U_3-R-U_5 by transcription of "plus strong

stop DNA". The plus strand acquires VIRAL GENOME-U_3-R-U_5 by transcription of minus strand DNA. The final product is double stranded DNA with two copies of U_3-R-U_5 (the LTR).

This model has been proposed by Gilboa et al. [37]; similar models have been proposed by others [28,67]. Experimental evidence in support of the model was obtained by exploiting the ability of actinomycin D to specifically inhibit DNA-dependent DNA synthesis, thus enabling investigators to distinguish between plus and minus DNA transcripts. The existence of minus "strong stop DNA" has been well documented; recently "plus strong stop DNA" has been identified as a reverse transcription intermediate in many retrovirus systems [10,28,79,93]. Reverse transcription of the 3' end of the tRNA primer by the nascent plus strand has been shown in some avian and murine rertoviruses [37,132,137]. There is still some uncertainty as to whether the plus strand is synthesized as one continuous strand [37] or whether a strand displacement mechanism is involved [67].

Integration is highly specific with respect to the viral genome. The structure of the provirus is CELL DNA-U_3-R-U_5-VIRAL GENOME-U_3-R-U_5-CELL DNA. However, integration apparently occurs randomly with respect to the host genome (see chapter 1). The process of integration causes a 4-6 bp duplication of host sequences at the site of integration [27,56,63,86,121,124], a property retroviruses share with prokaryotic and eukaryotic transposable elements.

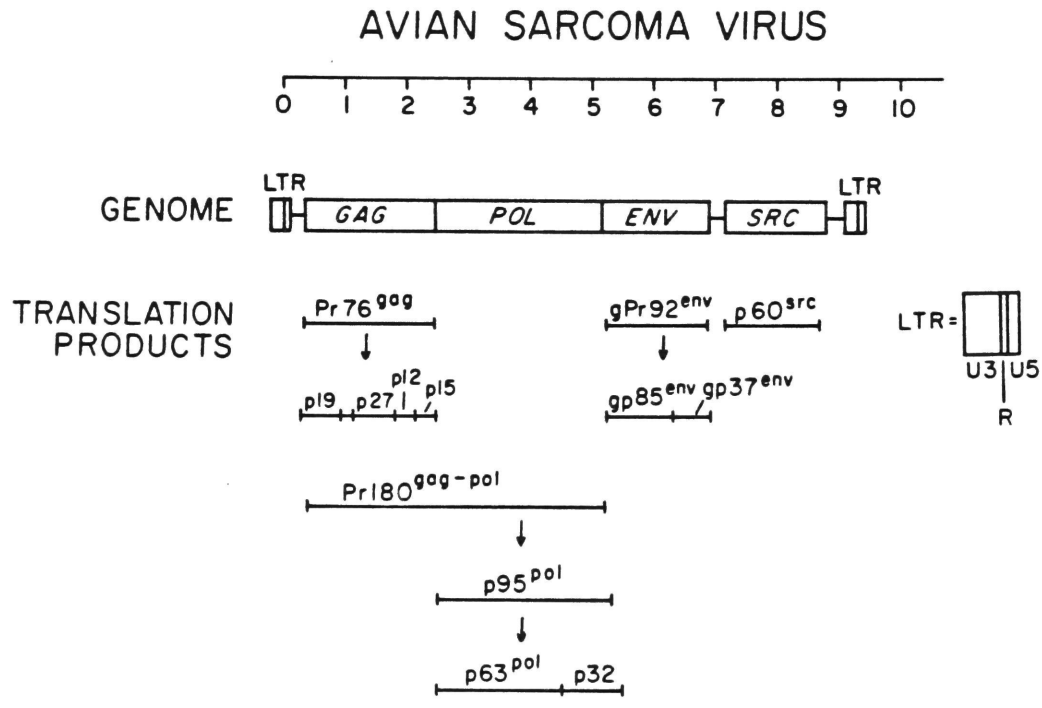


Figure 1. Genetic structure and protein products of non-defective avian retroviruses. The structure shown is that of an RSV provirus; except for the src gene, the remainder of the genome is the same in ALV. The primary translation products of viral mRNAs and their mature cleavage products are shown beneath the DNA sequences which code for them. Recently, a fifth gag protein, p10, has been mapped to the region between p19 and p27 [103].

Slowly Transforming Viruses:

Genetic Structure. The prototype slowly transforming viruses are the avian and murine leukosis viruses (ALV and MuLV). ALVs include Rous associated viruses (RAV) and related viruses. RAV-1 and RAV-2, commonly used laboratory strains, were originally isolated as replication competent "helper" viruses in defective Rous sarcoma virus (RSV) stocks [41,109]. These viruses contain 3 genes whose products are both necessary and sufficient for viral replication: gag, pol, and env (Fig. 1).

The gene order, gag-pol-env seems to be invariant. The LTR (U_3-R-U_5) is present on both ends of the proviruses. In addition to the three coding regions, there are non-coding sequences upstream from gag and downstream from env.

Studies of the DNA of normal uninfected chickens demonstrated the existence of at least 16 genetic loci (numbered ev-1 to ev-16) containing endogenous viral sequences, structurally related to ALV. Most chickens of the White Leghorn breed (which is commonly used in laboratory studies) seem to contain at least the ev-1 locus; usually one to three additional ev's are also present. Most ev's have multiple defects and are thus unexpressed; some ev's express part of gag ("gs^+" phenotype) and/or env ("chf^+" phenotype) [53].

Transcription and Translation: The gag gene codes for the viral structural proteins, p19, p10, p27, p12, and p15. Genomic-sized mRNA is translated into a 76 kD gag polyprotein precursor product ($Pr76^{gag}$), which undergoes proteolytic

cleavage to generate the five viral proteins. Processing occurs in the infected cells and pulse-chase experiments have demonstrated a presursor-product relationship between Pr76 and the mature gag proteins [51,100].

The pol gene codes for reverse transcriptase. In the avian system the active enzyme is a dimer consisting of one molecule of an alpha subunit, $p63^{pol}$, and one molecule of a beta subunit, $p95^{pol}$. p95 occupies most, if not all, of the pol coding sequences [117], and p63 is an amino terminal cleavage product of p95 [34]. The carboxy terminal cleavage product of p95 is a 32 kD phosphoprotein (pp32, or p32) which has DNA endonuclease activity [115]. The exact role of the p32 endonuclease in replication has not been determined.

The identity of the mRNA species used for translation of pol remains an enigma. The primary translation product of pol is a 180 kD polyprotein precursor shown to have gag and pol antigenic determinants and to contain the tryptic peptides of $Pr76^{gag}$ and $p95^{pol}$ [100,105]. Pr180, however, is not a precursor to Pr76 since radioactivity from Pr180 does not "chase" into Pr76 and since there is 20-50 times more Pr76 than Pr180 in cells even at early time points [100]. Pr180 was therefore considered to be a read-through protein resulting from occasional suppression of the gag termination codon used in synthesis of Pr76. However, in vitro translation in the presence of tRNA amber suppressor molecules yielded an 80 kD protein at the expense of Pr76 and did not result in an increase in Pr180 [147]. Recent DNA sequencing revealed the surprising fact that

gag and pol were encoded on different reading frames on viral DNA. The most plausable explanation for the synthesis of Pr180 requires that a splicing event removes the gag termination codon and 10 to 20 bp in the vicinity of the gag-pol juncton, resulting in a shift to the -1 reading frame while avoiding creation of new in-frame termination codons. The presence of a spliced pol mRNA has yet to be demonstrated. If such a molecule exists, a percentage of virion particles may contain this RNA instead of genomic RNA since in vitro translation of virion RNA resulted in the synthesis of Pr180 and Pr76 in practically the same ratio as found in infected cells [147].

The precursor-product relationship between Pr180 and p95/p63 has been inferred from pulse-chase experiments in which the kinetics of disappearance of Pr180 from infected cells correlates extremely well with the kinetics of appearance of p95 and p63 in virion particles [100]. Since p95 and p63 do not appear in immunoprecipitates of infected cells and since Pr180 has not been convincingly shown to be present in virion particles, it was not possible to demonstrate a direct relationship. Preliminary evidence presented in this thesis indicates that it may be possible to demonstrate a direct precursor-product relationship in the virion particles.

The env gene codes for the viral glycoproteins, gp85 and gp37. A 21S subgenomic spliced mRNA is translated into a glycosylated precursor, $gPr92^{env}$, the major viral glycoprotein detected in infected cells. In the presence of inhibitors of glycosylation, this precursor runs as a 57 kD unglycosylated

protein. The product of cell-free translation of 21S mRNA runs as a 63 kD unglycosylated protein, presumably due to the presence of a signal peptide in the in vitro synthesized product. Cleavage of gPr92 to gp85 and gp37 and formation of disulfide linkages between the two *env* proteins occurs near the time of budding, although the exact sequence of events is not clear.

$gp85^{env}$ confers subgroup specificity upon the virion particle. There are 6 defined viral subgroups, designated A-F, which are encoded in different viral gp85 genes. Since host cells may contain receptors on their surfaces for only some of these 6 subgroups, host range is also related to gp85. Because of variations in subgroup, the gp85 portion of *env* is the part of the genome that displays the most variability among various viral strains.

The leader sequence of the spliced *env* mRNA contains approximately 250 nucleotides from the 5' end of the genome. The proposed splice donor site is located at nucleotide 18 of the Pr76 coding sequence; the proposed splice acceptor site for *env* is found within the coding sequences for an *env* signal peptide. This implies that the signal peptide is composed of amino acids which are partially derived from the leader sequence (i.e., the first 6 amino acids). Interestingly, DNA sequencing has shown that the coding sequences of *pol* and the *env* signal peptide overlap by ~110 nucleotides with *pol* and *env* in different reading frames. The actual coding sequences do not overlap; *env* begins 56 bp after *pol* terminates.

Rapidly Transforming Viruses:

Rous Sarcoma Virus. Rapidly transforming viruses are characterized by the presence of a specific onc sequence inserted into their genomes in addition to, or instead of, the replicative genes. The most extensively studied virus of this class has been RSV (Figure 1).

In RSV, gag, pol and env are present and are transcribed and translated in the same manner as described for ALV. The size of the env mRNA of RSV is 28S, since both env and src are transcribed; however only env is translated from this mRNA.

In addition to the ALV genes, RSV has a sequence known as src inserted into its 3' end, between env and the LTR. Oncogenicity and transformation in tissue culture can be directly attributed src since 1) ALV and RSV, which have quite different pathologies, are essentially identical except for this sequence, and 2) spontaneously-derived deletion mutants in src are invariably transformation defective (td) and have lost the ability to cause rapidly appearing tumors in vivo [70]. The isolation of mutants which were temperature sensitive for transformation [72,89] suggested that the src gene product was a protein. Finally, Erikson et al identified a 60K product of the src gene which had an associated protein kinase activity [35,36].

Unlike the other viral proteins, src is not a cleavage product of a larger protein precursor. $p60^{src}$ is the primary translation product of a separate spliced subgenomic mRNA of 21S.

Other Rapidly Transforming Viruses. Based on the genetic

structure of RSV, the prototype transforming virus was considered to be composed of gag-pol-env-onc; transforming viruses which were replication defective (i.e., lacked gag, pol and/or env information) were considered to be exceptions to this rule.

The first defective transforming virus to be described was the env-defective Bryan high titer strain of RSV (BH-RSV) [45]. It has subsequently become clear that, except for certain strains of RSV, all rapidly transforming viruses are defective in replication. One or more replicative genes are replaced by a new sequence containing the onc gene. Cells containing such viral genomes are transformed but do not release infectious viral particles ("non-producer" cells). Figure 2 compares the structures of some rapidly transforming viruses with that of ALV.

The structures of the defective viruses range from replacement of one gene (BH-RSV and AMV) to replacement of all replicative genes (Ha-MSV). In viruses in which the onc gene is inserted into gag, the transforming gene product is expressed as a gag-onc fusion protein which is synthesized from a genome-size mRNA. Products of transfoming genes in more distal positions (such as AMV myb and AEV erbB) are thought to be synthesized off subgenomic spliced mRNA transcripts. The src gene product of BH-RSV is known to be synthesized off a 21S mRNA similar to non-defective RSV.

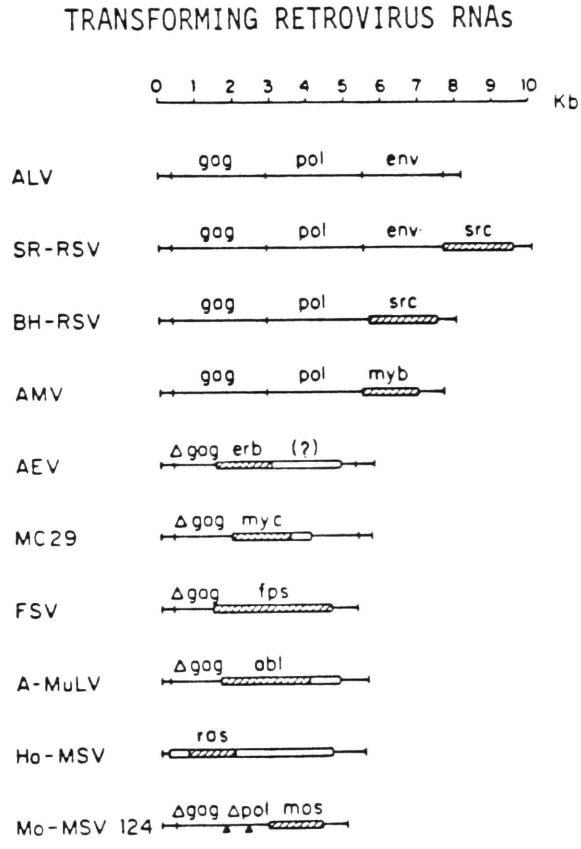


Figure 2. Genetic maps of some representative viruses. Inserted sequences, apparently of cellular origin, are shown in boxes. Sequences which code for transforming functions (indicated by cross-hatching) are labeled according to the currently accepted nomenclature [17]. (In AEV the region identified as erb has recently been renamed erbA and the region identified by the question mark has been named erbB.)

In order to replicate, defective transforming viruses require that the missing viral products be supplied by a replication competent helper virus (such as ALV). The resulting virion particle is called a pseudotype; the physical particle is derived partly or wholly from the helper virus and the RNA genome is from the defective virus.

Origin of the onc Sequences. Stehlin et al. discovered that cDNA specific for src hybridized to cellular DNA of normal uninfected chickens with kinetics which indicated that one to two copies of a cellular src (c-src) gene were present [130]. Hanafusa et al. found that upon injection of partial td mutants of RSV into chicks, tumors arose after a long latent period from which a non-defective RSV could be recovered [43]. Studies of the RNAse T_1 oligonucleotide fingerprints [146] as well as tryptic peptide analyses [68] revealed that the src genes of these recovered avian sarcoma viruses (rASV) were the products of recombination between the residual src of the partial td mutant and the c-src gene.

Subsequently, at least 13 more unique sequences, all of which have cellular homologs, have been identified as the transforming genes in different rapidly transforming retroviruses. These findings led to the, now generally accepted, hypothesis that transforming retroviruses arose by recombination events between helper viruses and c-onc genes. The level of expression and/or the structure of the c-onc genes seem to have been altered as a result of this recombination, endowing these normal genes with potent transforming abilities.

Early History of RSV: Origin of the Common Laboratory Strains. In 1911 Peyton Rous of the Rockefeller Institute reported the isolation of a filterable agent which produced sarcomas in chickens [107]. The filtrate originated from a spontaneous tumor in a Plymouth Rock hen; it was designated "Chicken tumor #1" by Rous, and later became known as Rous sarcoma virus. The tumor had been passaged for a few years by transplantation in blood relatives of the original chicken before it acquired the ability to grow in all breeds of chickens. After the discovery of the "filterable agent", virus was routinely passaged by inoculation into chickens and recovery from the resulting tumors.

The virus was passaged by Rous and his collaborators in New York and samples were given to researchers at other institutions. However, the passage history of the virus was not well documented. RSV strains available today are all considered to be derived from the original RSV, but precise data on the evolution of each strain is not available. Until the 1960s, when tissue culture methods became popular, RSV was propagated by in vivo passage. Since it is now known that in vivo passage can lead to the generation of novel recombinants [1,43,49], the relationship between the modern RSV strains and the original isolate is not clear. Unfortunately, there are no remaining samples of the tumor material isolated by Rous.

Most research in the past twenty years has been performed on a few strains, named after researchers involved in their propagation. 1) The Schmidt-Ruppin strain of RSV (SR-RSV) was used extensively by K.H. Schmidt-Ruppin of Germany in the

1950s. His material originated from Rous' tumor; however it was passaged in two other laboratories before he received it in 1953 [95]. 2) The Prague strain of RSV (PR-RSV) was used by J. Svoboda of the Institute of Biology in Prague in the late 1950s. The genealogy of this strain is not clear. 3) The Bryan high titer strain of RSV (BH-RSV) was developed by W.R. Bryan of the National Cancer Institute in 1959 as a result of over 50 successive passages of virus in New Hampshire Red chickens. At each passage Bryan selected for the most rapidly growing tumor to use as material for the next round of inoculation [14]. Bryan originally received RSV in 1941 from A. Claude of the Rockefeller Institute; Claude received it from J. Murphy, a collaborator of Rous [95]. 4) The Bratislavia 77 strain (B77) of avian sarcoma virus was isolated as a spontaneous field isolate [141]. The original B77 stock may have been lost to contamination or mislabeling, since the present day "B77" is indistinguishable from PR-RSV.

The Bryan Strain of Rous Sarcoma Virus. Hanafusa et al. showed that BH-RSV was defective in the production of infectious virus and required a helper virus for replication [41,45]. The defect could also be overcome by passaging the virus in cells of certain chicken embryos. These embryos produced a "chicken helper factor" (chf) which rendered the BH-RSV virions infectious for one round of replication on quail but not chicken cells. (It was later shown that chf was subgroup E viral glycoprotein; quail cells have receptors for this subgroup, whereas chicken cells generally do not).

Biochemical studies showed that BH-RSV virions lacked the viral glycoproteins, gp85 and gp37 [114]. Oligonucleotide mapping detected the presence of a large deletion in BH-RSV as compared to non-defective RSV [30]; the position of this deletion thus defined the location of *env* on the genetic map. BH-RSV was used extensively as the prototype *env*$^-$ mutant to elucidate the function of the *env* gene products, to identify the *env* mRNA species, and to use as a negative selection for the preparation of *env*-specific DNA probes. However, the precise extent of the *env* deletion had not been determined and it was not known whether any residual *env* information was present in BH-RSV.

Hanafusa et al observed that 5-10% of viral particles in BH-RSV stocks could not be complemented by passaging through chf$^+$ cells [46]. These particles, termed BH-RSV alpha, were found to have an additional defect: reverse transcriptase activity could not be detected in these virions [44,47,101,148]. The *pol* defect was extremely stable; reversion of the *pol*$^-$ phenotype to *pol*$^+$ was not observed. However, upon superinfection of BH-RSV alpha-infected cells with helper virus, recombination occurred, resulting in the formation of the parental *pol*$^+$ BH-RSV (BH-RSV beta). BH-RSV alpha is also capable of recombining with a *pol* mutant, tsLA337, to yield *pol*$^+$ BH-RSV beta, but incapable of recombining with another *pol* mutant, tsNY21 [111]. This would suggest that the defect, most likely a deletion, maps to a particular region of *pol*. RNA sizing studies failed to detect a difference between BH-RSV alpha and

the parental, BH-RSV beta [L-H Wang, personal communication]. The nature of this defect and the mechanism for its generation remained a puzzle.

Since BH-RSV alpha virion particles contain viral RNA, selection of genomic RNAs for packaging into virions does not seem to require functional reverse transcriptase. However, BH-RSV alpha virions (as well as those of another pol mutant) do not contain significant amounts of $tRNA^{trp}$ and the other small RNAs found avian retrovirus particles [104,113], thus implicating the reverse transcriptase protein in selection of tRNAs for packaging.

Scope of This Thesis: Because of their specific non-conditional defects, the BH-RSV strains have traditionally been excellent model systems for studying many viral functions. In this thesis I present results of experiments using BH-RSV alpha to study the specificity of RSV integration into its host's DNA. Spread of infectious virus and concomitant multiple integration events occurred when non-defective RSV was used; this prevented examination of specific proviruses and the adjacent cellular DNA. With BH-RSV alpha as the exogenous virus, bands representing the single integrated provirus were readily detected and the lack of specific integration sites was clearly determined.

I examined the structure and properties of the BH-RSV alpha and beta mutants in an attempt to fully characterize their defects. A DNA fragment containing the region flanking the env deletion in BH-RSV was sequenced, the nature of the pol protein

products encoded by BH-RSV alpha was determined, and the BH-RSV alpha defect was localized to a specific region of the pol gene. Based on sequence comparisons between BH-RSV and other retroviruses, I also obtained suggestive evidence for the importance of specific non-coding regions flanking the src gene of RSVs.

MATERIALS AND METHODS

Cells and Viruses:

A clone of 3Y-1 rat cells infected with BH-RSV was obtained from S. Kawai and grown in MEM media as described [69]. A stock of a RAV-1 pseudotype of BH-RSV alpha virus, [BH-RSV alpha (RAV-1)], was generously provided by T. Hanafusa. All preparations of BH-RSV alpha (RAV-1) are known to contain a small percentage of the pol^+ BH-RSV beta (RAV-1) which is considered to be a consequence of recombination events which occurred after superinfection with helper virus [46]. BH-RSV alpha-transformed chicken cells were grown in Scherer's or Hams' F-10 medium as previously described [42]. CEF were from gs^-, chf^- SPAFAS embryos.

Viral Assays:

The presence of infectious transforming virus was assayed by ability to form infectious centers on uninfected CEF in a standard hard agar focus assay [140]. Viral reverse transcriptase activity on an exogenous template (poly rC:oligo dG) was measured under standard conditions [145].

Cloning BH-RSV Infected "Non-Producer" Chicken Cells:

Clonal populations of chicken cells harboring BH-RSV proviruses were obtained by infecting CEF with BH-RSV alpha (RAV-1) at high virus dilutions (m.o.i. 10^{-5}-10^{-6} focus forming units/cell). Within 24 hours post-infection cells were overlaid with agar medium containing antibody that neutralized

RAV-1. Individual foci picked on day 8 were cultured separately, and were continuously monitored for morphology, infectivity, and virion polymerase activity. Clones of cells that were morphologically transformed, but did not produce infectious virus as assayed by focus formation, were further monitored for the production of virus particles by sucrose gradient banding of [^{35}S]-methionine labeled particles from labeled cultures [106]. Transformed clones producing noninfectious virions were designated as BH-RSV clones. Those BH-RSV clones which, in addition, released polymerase-negative particles were designated BH-RSV alpha clones; the clones in which sedimentable polymerase activity was detected were designated BH-RSV beta clones. [BH-RSV beta clones have also been referred to in previous studies as BH-RSV(-)].

DNA Extraction:

DNA was extracted from pellets of 10^8 cells by the procedure of R. Junghans, Roche Institute (personal communication). The cell pack was lysed in extraction buffer containing 70% guanidine thiocyanate, 10% 2-mercaptoethanol, 0.02 M Tris (pH 8), and 0.001 M EDTA. CsCl crystals were added to a final concentration of 1.4 g/ml and the final volume was adjusted to 2 ml with extraction buffer. The lysate was layered on top of a preformed CsCl step gradient: 1.8 g/ml and 1.6 g/ml CsCl solutions in TEN buffer (0.01 M Tris (pH 7.4), 0.001 M EDTA, and 0.01 M NaCl). DNA was banded for 72 h at 34K in a fixed angle rotor (Beckman T40 or Ti50) and the DNA-containing fractions, identified by viscosity and/or agarose gel electro-

phoresis with ethidium bromide staining, were dialyzed against TEN buffer for a minimum of 5 buffer changes (at least 8 h per buffer change). Dialyzed DNA was then used directly in restriction enzyme reactions.

Enzymes and Enzyme Reactions:

Restriction endonuclease reaction conditions were as per manufacturer's (New England Biolabs or Bethesda Research Laboratories) directions; enzyme buffers were either as recommended by the manufacturer or as described in Davis, et al. [25]. Usually, at least a twofold excess over the recommended amounts of enzyme was used for digestion of chromosomal or closed circular plasmid DNA.

Digestions of chromosomal DNA were monitored by incubation of an aliquot (usually 5%) of the chromosomal reaction mixture with 1 ug of lambda or Ad-2 DNA. This marker DNA was subjected to electrophoresis on an agarose gel, stained with ethidium bromide, and examined under UV light for completeness of digestion. Chromosomal samples were precipitated in ethanol and run on agarose gels only after completeness of digestion was demonstrated. Digestions of phage or plasmid DNA were monitored directly on agarose gels.

Nick translation was performed as described by Maniatis, et al. [88]. The labeled nucleotide was [alpha ^{32}P]-dCTP, (specific activity: >2000 Ci/mmole) 10 mCi/ml in aqueous solution (Amersham) and DNA polymerase I was obtained from Boehringer-Mannheim; specific activities of 10^8 cpm/ug DNA were routinely obtained.

Ligation of DNAs was performed using under conditions recommended by the manufacturer for joining fragments with cohesive ends. Reactions contained 1 ul (10 units) of T_4 DNA ligase (New England Nuclear) in the recommended buffer (50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 50 ug/ml BSA, 1 mM ATP, 10 mM DTT) and were performed at 16°C for 3-12 hours. Ligation of lambda arms to insert DNA required a high DNA concentration in the reaction mixture (>100 ug/ml); ligation of plasmid and insert fragments to form circular products required more dilute DNA concentrations (10-50 ug/ml).

Removal of 5' phosphate from DNA fragments was accomplished by bacterial alkaline phosphatase (Bethesda Research Laboratories) treatment at 68°C for 1 hr in 10-50mM Tris-HCl (pH 8.0).

5' end-labeling of DNA fragments with T_4 polynucleotide kinase was performed at 37°C for 30 min. Reaction volumes were 40 ul and contained between 0.5 and 10 pmoles of available 5' ends and 40 pmoles of [gamma-^{32}P]-ATP (Amersham, specific activity 2000-3000 Ci/mmole, 10 mCi/ml in aqueous solution) in kinase buffer (50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA).

Gel Electrophoresis of DNA and Filter Hybridization:

For agarose gel electrophoresis, DNA samples were run on submerged horizontal gels (20 x 25 x 0.5 cm or 8.5 x 5.5 x 0.5 cm "mini-gels") in electrophoresis buffer (E buffer) (0.04 M Tris-acetate (pH 7.8), 0.005 M Na-acetate, and 0.001 M EDTA). The agarose concentration was usually 0.6-0.8% and size markers

were lambda HindIII fragments; however when small (less than 1 kb) DNA fragments were examined, agarose concentrations of 1.5-2% and size markers of pBR322 HinfI fragments were used. DNA was visualized by ethidium bromide (0.5 ug/ml) staining and UV light excitation.

For sizing restriction fragments smaller than 1 kb or as a preparative step in obtaining a labeled DNA fragment for sequencing, electrophoresis on polyacrylamide slab gels was performed. The concentration of polyacrylamide was 5 or 8% (the ratio of acrylamide to bisacrylamide was usually 19:1, although in some cases 50:1 ratios were used) in TBE buffer (50 mM Tris (pH8.3), 50 mM boric acid, 1 mM Na_2EDTA) as described by Maxam and and Gilbert [92]. For both agarose and polyacrylamide gel electrophoresis, the voltage and the time of electrophoresis was usually determined empirically.

Southern transfer [127] of DNA from agarose gels to nitrocellulose filters (Schleicher & Schuell), hybridization to radioactive probes, and washing of filters were carried out as previously described [10, see also Molecular Cloning section below], except that unlabeled salmon sperm carrier DNA was added to the hybridization solution at a concentration of 250 ug/ml. Filters which were to be re-hybridized to a second radioactive probe were soaked in pre-hybridization solution (hybridization solution with H_2O substituted for formamide) at 80°C for 10 min to remove the first probe.

Radioactive Probes:

1. RAV-2_{rep} probe: RAV-2 DNA purified from a lambda RAV-2 clone [65] was cut into two fragments with the enzymes HindIII and SalI. Such cleavage generates one fragment of 4.25 kb, which contains a portion of the env gene, the LTR, and the entire gag gene, and another fragment of 3.3 kb, which contains the entire pol gene and part of the env gene. These two fragments have been subcloned separately into pBR322 [133]. [^{32}P]-RAV-2_{rep} probe was prepared by nick translation of a 1:1 mixture of these two pBR322 subclones.

2. pol probe: [^{32}P]-pol probe was prepared by nick translation of the pBR322 subclone containing the 3.3 kb HindIII-SalI RAV-2 fragment mentioned above.

3. $cDNA_{5'}$: [^{32}P]-$cDNA_{5'}$ which consists of the first 101 nucleotides from the 5' end of RAV-2 (formerly known as "strong stop" DNA), was generously provided by B. Neel, The Rockefeller University. This probe was made by previously published procedures [50,53].

4. LTR probe: [^{32}P]-LTR DNA which contains all the 5' and 3' sequences present in one copy of the long terminal repeat of SR-RSV was obtained by EcoRI digestion of a lambda SR-RSV clone which contains two copies of the LTR [133] followed by nick-translation of the purified 350 nucleotide fragment.

5. src probe: [^{32}P]-src probe was made either by nick translation of a pBR322 subclone containing the 3.8 kb EcoRI src-containing restriction fragment from the lambda SR-RSV clone

mentioned above [133], or by nick-translation of a purified 600 bp HaeIII fragment cut from the 5' region of the src gene of the subclone.

DNA Sequencing and Analysis of Sequence Data:

The chemical cleavage method of Maxam and Gilbert [92] with the hydroxylamine modification of Schmid and Rubin [108] for cleavage at cytosines, was used throughout. Fragments to be radioactively labeled were generated by cleavage with restriction enzymes which produce 5' protruding ends. The 5' ends were radioactively labeled using T_4 polynucleotide kinase and gamma-[^{32}P]-ATP as described above. To obtain DNA fragments labeled at a single end, further digestion of double end-labeled fragments with other restriction enzymes or separation of the two DNA strands was performed; both procedures are described by Maxam and Gilbert [92]. All regions were sequenced at least twice (on both DNA strands, when possible); the sequences of restriction enzyme cleavage sites were confirmed by DNA sequencing.

Analysis of DNA sequences for the presence of restriction enzyme cleavage sites, or for identification of regions of extensive homology was facilitated by use of the computer programs SEARCH and SEQFIT, respectively [128].

Bacterial Cells, Phages, and Plasmids:

Escherichia coli strain ED 8654 [96] was used for isolation and propagation of recombinant bacteriophage. The bacteriophage vector lambda gtWES lambda B [82] was used to con-

struct recombinant bacteriophage. This vector was digested with Eco Rl and Sac I, and the Eco Rl arms were purified from the dispensable lambda B fragments by sucrose density gradient centrifugation. E. coli strain HB101 was used for transformation and amplification of plasmids [12]. The plasmid pBR322 [9] was used in the construction of recombinant plasmid DNA.

The recombinant plasmid clones pSR-REP and pSR-XD were generous gifts of F. Cross, The Rockefeller University. The construction of these plasmids from pSRA [26] has been described [24].

Molecular Cloning of Fragments from BH-RSV Proviral DNA:

550 micrograms of pooled DNA from BH-RSV alpha chicken cell clones and 350 micrograms of DNA from BH-RSV beta rat 3Y-1 cells were completely digested with EcoRI. Digested DNA was fractionated on a sucrose gradient, and fractions containing the 5 kb viral pol-src fragment were identified by subjecting an aliquot of each fraction to agarose gel electrophoresis and subsequent hybridization of the Southern blot to src-specific probe. For both BH-RSV alpha- and BH-RSV beta-containing DNA, the hybridizing band was present in three consecutive fractions; DNA from the fraction with the strongest hybridization was concentrated by precipitation in ethanol. The yield was approximately 30 and 10 micrograms of 5 kb insert from BH-RSV alpha and BH-RSV beta, respectively. 0.075 micrograms of insert DNA was ligated to 1 microgram of EcoRI arms of the phage vector, lambda gt WES, (2:1 molar ratio of arms:insert) and packaged in an in vitro lambda packaging system [57].

Details of procedures for in vitro packaging [5,57], screening [6], phage purification, amplification and preparation of phage DNA have been previously described [65]. The efficiencies of packaging of recombinant bacteriophage ranged from 10^5 to 10^6 infectious particles/ug phage DNA. Recombinant bacteriophage were plated on 20 x 20 cm "bioassay" plates (Nunc) (200,000-400,000/plate) and screened. Before hybridization, filters containing phage DNA imprints were prewashed at 37° in pre-hybridization buffer (5 X SSC (1 X SSC is 0.15 M NaCl, 0.015 M Na citrate), 0.02 M Tris-HCl (pH 7.4), and 0.5% SDS) for at least 45 min. Hybridization was was carried out at 37° in hybridization buffer (50% formamide, 5 X SSC, 20 mM Tris-HCl (pH 7.4), 0.5% SDS), and at least 200 ug/ml salmon sperm DNA for 18-24 hr. Ten ml of hybrid mix containing 2 x 10^6 Cerenkov cpm of probe were used for each filter. Following hybridization, filters were washed 3 times briefly in 0.2 X SSC/0.2% SDS and then incubated once or twice for 45 min each at 37° in the same solution. Positive plaques were purified three times before amplification.

Purification of DNA Fragments:

DNA fragments visualized by ethidium bromide staining of agarose or polyacrylamide gels were purified as a result of being cut out from the gel and electroeluted in dialysis tubing containing E buffer.

Subcloning in Plasmid Vectors:

The 5 kb EcoRI fragment from BH-RSV alpha and BH-RSV beta

was purified from the respective lambda clones and subcloned into pBR322 according to standard procedures: phosphatase treated EcoRI-cut pBR322 was ligated to equimolar amounts of 5 kb insert DNA and the ligated products were used to transform E. Coli HB101. For in vitro recombination reactions, insert and vector fragments (described in the text) were ligated and the ligation products were used to transform HB101.

Transformation of E. Coli was accomplished by addition of ligated plasmid DNA to $CaCl_2$-treated bacterial cells [87]; transformants were identified by their ability to form colonies on agar media containing tetracycline (EcoRI inserts) or ampicillin (EcoRI or SalI inserts). For initial screening of clones, plasmid DNA of antibiotic-resistant colonies was isolated from 1-5 ml bacterial cultures by the rapid alkaline lysis technique [8].

Transfection of CEF with Viral DNA:

Preparation of CEF and transfection of SR-RSV cloned DNA were performed as described [24]. Secondary CEF were seeded 16-24 hr prior to transfection at a density of 7×10^5 cells/60 mm culture dish. For the transfection period only, the culture medium was replaced with F10 medium lacking Tryptose Phosphate broth. Equal quantities (between 0.1 and 1 ug) of pSR-REP DNA (or pSR-REP-derived recombinants) and pSR-XD DNAs were cut with SalI, ligated briefly (30 min at $16^{\circ}C$, in standard ligation buffer with 2 units of T_4 ligase in 50 ul, at a DNA concentration of approximately 200 ug/ml) and transfected onto CEF. The DNA-calcium phosphate coprecipitate remained on the cells for 6

hr; at 6 hrs and again at 18 hrs, the culture supernatants were replaced with fresh F10 medium. Cells were passaged on the third day post-transfection, overlaid with F10 soft agar medium 24 hrs later, and monitored for the appearance of characteristic foci. For determination of the titer of infectious viral particles in culture supernatants, agar overlay was omitted.

Isotopic Labeling of Cells and Preparation of Cell Extracts:

Unless otherwise indicated, cells grown in 65-mm tissue culture plates were incubated for 4 hr in 1 ml of methionine-free minimal essential medium (GIBCO) containing 100 uCi of L-^{35}S-methionine (1200 Ci/mmole, Amersham or New England Nuclear). The cultures were washed twice in cold Tris-Glu buffer (25 mM Tris pH 7.4; 135 mM NaCl; 5 mM KCl; 0.4 mM Na_2PO_4; 5.5 mM glucose), and lysed in 400 ul in slightly modified RIPA buffer [13], (0.05 M Tris-HCl, pH 7.4; 0.15 M NaCl; 1% Trition X-100; 1% sodium deoxycholate; 0.1% SDS; 1 mM EDTA) containing 1% Trasylol (a general serine protease inhibitor, FBA Pharmaceutical, New York). Lysed cells were scraped from the plate with a rubber policeman and transferred to a 1.5 ml Eppendorf micro test tube (Brinkman). After vigorous vortex mixing for 30 sec, the sample was centrifuged in a precooled Beckman Microfuge B for 4 min. The resulting clarified supernatant was used in all subsequent manipulations (100 ul per immunoprecipitation).

Isotopic Labeling of Virus and Preparation of Virion Extracts:

For continuous labels, transformed cells grown in 65-mm

tissue culture plates were incubated in 1 ml of methionine-free minimal essential medium (GIBCO) containing 100 uCi (unless otherwise indicated) of L-[^{35}S]-methionine for two successive 12 hr labeling periods followed by a 12 hr incubation in complete medium. Culture supernatants from the three incubations were harvested and cell debris was removed by pelleting in a Sorval centrifuge (8,000 rpm for 20 minutes). To obtain purified labeled virions, the clarified supernatant was layered over 2 ml of 20% sucrose in a Beckman SW50.1 centrifuge tube. Centrifugation at 45,000 rpm for 45 minutes resulted in precipitation of virion particles. The virus pellet was resupended in 100 ul of RIPA buffer containing Trasylol (1%) and used in immunoprecipitation experiments.

For pulse-chase experiments, the labeling times and amounts of isotope used are described in the figure legend. Unless otherwise indicated, purification of the labeled virions was performed as described above.

Immunoprecipitation:

Rabbit antiserum raised against total virion proteins (anti-virion serum) was a generous gift from Ricardo Feldman, The Rockefeller University; antiserum raised against avian myeloblastosis virus reverse transcriptase (anti-pol serum) was obtained from the Office of Program Resources and Logistics of the National Cancer Institute. Preabsorbed serum was prepared by adding 10-20 ug of protein from disrupted purified RAV-2 in modified RIPA for each 1 ul of anti-pol serum, incubating at 4^{o}C for 60 min, and spinning out the precipitate in a Beckman

Microfuge. Aliquots of cell extracts were incubated with 5 ul of unabsorbed serum or 10 ul of absorbed serum for 60 min on ice. Three serum volumes of Staphylococcus aureus protein A-Sepharose CL 4B (Sigma) (a 50% vol/vol slurry in modified RIPA buffer) was added and mixed for 30 min at 4° C to adsorb immune complexes by the method of Kessler [75]. The Sepharose pellet was washed five times in RIPA buffer and twice in H_2O. The pellet was then suspended in 40-100 µl of gel sample buffer (62.5 mM Tris (pH6.7), 3% SDS, 10% sucrose, 0.1 M DTT) and boiled for 5 min. before loading on a polyacrylamide gel.

SDS-Polyacrylamide Gel Electrophoresis:.

Polyacrylamide slab gels (ratio of acrylamide to bisacrylamide was 30:0.8) containing 0.1% SDS were prepared according to the procedure of Laemmli [80]. Gels were run at 12 to 20 mA constant current, until the tracking dye reached the bottom of the gel. Gels were fixed briefly and were stained when it was necessary to identify unlabeled molecular weight markers. To locate [^{35}S]-methionine-labeled proteins, fixed gels were fluorographed using Enhance (New England Nuclear) according to the manufacturer's directions. Gels were dried onto filter paper before exposure to film.

Chapter 1

Integration of RSV into chicken cellular DNA

INTRODUCTION

Since the integrated proviral DNA of retroviruses is a linear non-permuted copy of the viral RNA with LTRs at both ends [18,38,62,111], the integration event seems to be highly specific with respect to the viral DNA. In this study, BH-RSV alpha-infected CEF were used as a model system to determine whether there was any specificity for proviral integration on host DNA.

Although the techniques used in this study of proviral integration were similar to the methods used in the other studies of this nature, attempts were made to overcome two difficulties encountered in previous analyses of ASV-infected chicken cell clones [61,111]: 1) the ambiguity in identification of fragments corresponding to exogenous proviruses, and 2) the lack of clonality of the exogenous proviruses found in chicken clones grown by conventional methods. The first problem was addressed by including in our DNA analyses the uninfected CEF from which the infected clones were derived. The second problem was overcome by the use of BH-RSV alpha as the exogenous provirus.

The lack of clonality of exogenous proviruses within a colony of ASV-infected chicken cells is likely due to virus spread and reintegration. Since the BH-RSV alpha strain is defective in the functioning of both the *pol* and *env* genes [46,47], the virions are therefore non-infectious. A cell in-

fected with highly diluted BH-RSV alpha should divide and grow into a clone in which each cell contains a provirus only at the original integration site(s). Therefore, use of this defective RSV allows one to examine cells containing one (or few) proviruses without resorting to the use of non-permissive heterologous host cells. Analysis of such BH-RSV alpha-infected chicken cell clones showed that, in fact, clonality of proviral integration sites was maintained.

The results presented here demonstrate that expression of the viral genes could occur after proviral integration at many sites on the chicken genome and that there was no apparent preference for specific integration sites.

RESULTS

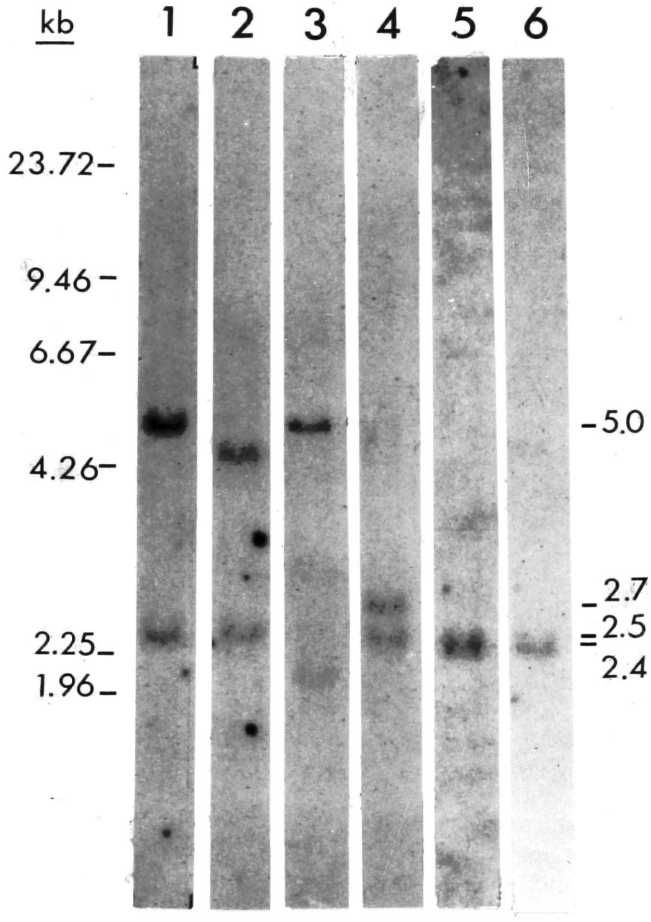
Restriction Map of BH-RSV

To determine which restriction enzymes would be suitable in studying of BH-RSV alpha integrated proviruses and in preparation for eventual molecular cloning of this virus, a preliminary restriction map of the provirus was constructed. For this purpose I used a clonal line of 3Y-1 rat cells which contained integrated provirus from BH-RSV beta [69] as a source of DNA.

EcoRI sites in BH-RSV. *EcoRI* cuts within the LTR of Rous sarcoma and avian leukosis viruses [26,38,54,58,65,118,139]; therefore, this enzyme is very useful for mapping studies since it generates the same internal proviral fragments regardless of the cellular site of integration.

The *EcoRI* site in the *env* gene of other Rous sarcoma and avian leukosis viruses was not present in BH-RSV. Digestion of BH-RSV-infected 3Y-1 rat DNA with *EcoRI* yielded two fragments of approximately 2.5 and 5.0 kb which hybridized to viral sequences (Fig. 1.1, lane 1). Similar digestion of SR-RSV DNA yields three fragments of 2.5, 3.8, and 3.2 kb. Since the BH-RSV 2.5 kb fragment comigrated with the 2.5 kb *EcoRI* fragment from cloned SR-RSV DNA, hybridized to $cDNA_{5'}$, and did not hybridize to *pol* probe (data not shown), this fragment appeared to be identical to the 2.5 kb *gag*-containing *EcoRI* fragment identified in other Rous sarcoma and avian leukosis viruses. The ~5.0 kb fragment appeared only in *EcoRI* digests of BH-RSV

Figure 1.1. BH-RSV-infected rat cellular DNA digested with restriction enzymes and hybridized to viral probes. Samples containing of 10 ug of total cellular DNA were digested with the restriction endonucleases specified below, subjected to electrophoresis on agarose gels, and transferred to nitrocellulose paper as described in the text. The blots were then hybridized to the viral probes specified below. The positions of the lambda DNA molecular weight markers run in a parallel lane are indicated on the left. Size values are expressed in kilobases. Lane 1, EcoRI digestion, hybridized to RAV-2_{rep} probe; lane 2, EcoRI-HindIII double digestion, hybridization to RAV-2_{rep} probe; lane 3, EcoRI-SacI double digestion, hybridization to RAV-2_{rep} probe; lane 4, EcoRI-KpnI double digestion, hybridization to RAV-2_{rep} probe; lane 5, EcoRI-KpnI double digestion, hybridization to LTR probe; lane 6, EcoRI-KpnI double digestion, hybridization to src probe. Lanes 5 and 6 were rehybridizations of the sample in lane 4.


kb
1
2
3
4
5
6
23.72
9.46
6.67
4.26
2.25
1.96
5.0
2.7
2.5
2.4

DNA, whereas the two fragments of 3.8 and 3.2 kb normally present in EcoRI digests of RSV DNA, did not appear in this virus strain. The 5.0 kb fragment hybridized to pol and src probes, and did not hybridize to $cDNA_5$, (data not shown); the size and genetic content of this new fragment is consistent with its being a fusion of the 3.2 and 3.8 kb fragments, minus the env sequences.

Mapping other enzyme sites in relation to EcoRI sites. For integration studies, restriction enzymes which cleave at only a limited number of sites within a provirus are useful. DNA extracted from the BH-RSV 3Y-1 rat cells was digested with HindIII, SacI, KpnI, and PvuI, and then hybridized to $RAV\text{-}2_{rep}$, src, or LTR probes. Digestion with HindIII, SacI, or KpnI produced two large fragments which were detected by both $RAV\text{-}2_{rep}$ and LTR probes (data not shown). Therefore, these fragments were provirus-cell junction fragments. No viral fragments other than those detected with LTR probe (i.e., internal fragments) were detected with the $RAV\text{-}2_{rep}$ or src probe. This is consistent with the hypothesis that HindIII, SacI, and KpnI each have only one recognition site within the BH-RSV provirus.

The DNA was digested with EcoRI followed by a second digestion with HindIII, SacI, or KpnI. The fragments resulting from the double digestions were compared to those generated by single EcoRI digestion. The SacI site mapped within the 2.5 kb EcoRI fragment while the HindIII and KpnI sites mapped within the 5.0 kb fragment (Fig. 1.1, lanes 2-4). In the EcoRI-KpnI digestion, the 5.0 kb fragment was cleaved into a 2.7 kb frag-

ment which was detected with RAV-2_{rep} probe (lane 4) and a 2.4 kb fragment detected with LTR (lane 5) or *src* (lane 6) probe. A map of these enzyme sites on the BH-RSV genome is presented in Fig. 1.2. For the three enzymes other than *Eco*RI, the single recognition site in BH-RSV corresponded to a site mapped previously at the same position on the genome of other strains of RSV. However for *Hind*III and *Sac*I, a second site assigned to *env* in many RSV strains, was missing in BH-RSV.

The enzyme *Pvu*I cuts most RSV DNA in the U_3 sequences, generating a unit length provirus band upon hybrization to viral probe [26,38,58,65, 118,138,139]. Although the LTR of BH-RSV contained the *Eco*RI recognition sites, the *Pvu*I sites were absent (data not shown).

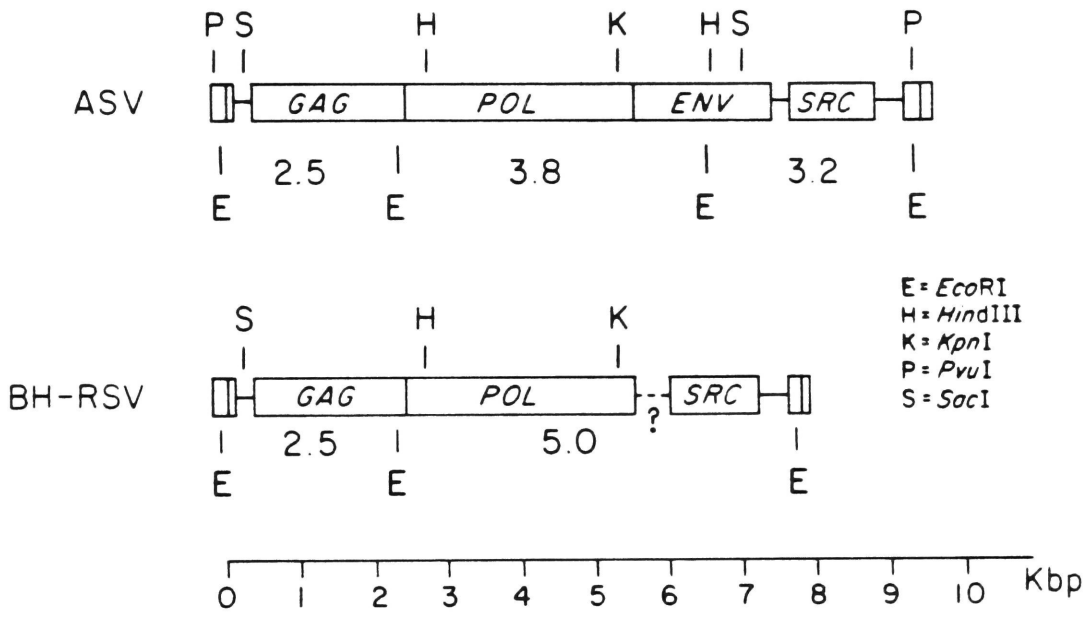


Figure 1.2. Restriction map of integrated proviral BH-RSV DNA compared with RSV DNA. The restriction enzyme cleavage sites on the BH-RSV genome were determined by single and double digestions, followed by hybridizations to specific viral probes as described in the text. Restriction enzyme sites on the RSV genome are shown for comparison. The RSV data were compiled from previous reports [26,54]. The sizes (in kilobases) of the EcoRI fragments of BH-RSV and RSV were determined by agarose gel electrophoresis. The broken line and the question mark on the BH-RSV genome represents the uncertainty regarding the amount of env information in BH-RSV. The boxes at the ends of the genomes represent the long terminal repeats of BH-RSV and RSV.

Analysis of BH-RSV alpha-Infected Chicken Cells: Integration Sites in Clonal Cells

Twelve independent "non-producer" clones were isolated from CEF infected with BH-RSV-alpha(RAV-1). Of the 12 clones, 11 were BH-RSV alpha type, 1 was BH-RSV beta; all 12 produced virion particles as well as all the intracellular viral precursor and structural (but not envelope) proteins (data not shown). DNA from the 12 clones and from the 2 uninfected embryos from which these 12 clones were derived was extracted and analyzed.

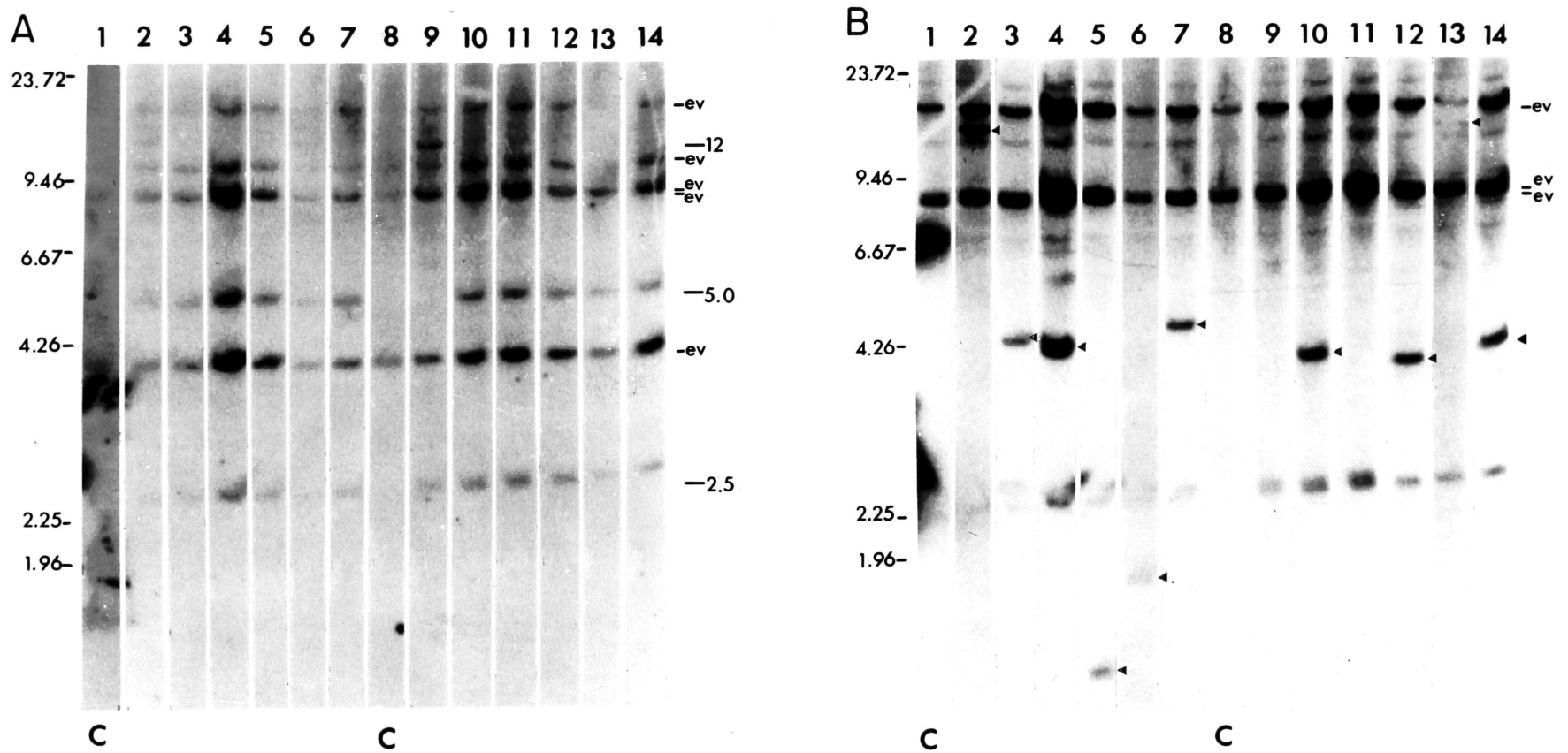
Integrity of the BH-RSV proviruses. DNAs from the 12 BH-RSV-infected CEF clones and the 2 uninfected chicken embryos were digested with EcoRI. Structural integrity of the proviruses was ascertained by the presence of the 2.5 kb and 5.0 kb internal BH-RSV EcoRI fragments.

Figure 1.3 (panel A) shows an analysis of cellular DNA cut with EcoRI and hybridized to RAV-2_{rep} probe. The RAV-2 probe was used to avoid detecting the cellular src bands. The same 5 bands of 15, 11, 8.6, 8.4, and 4.2 kb (the 8.6 and 8.4 kb bands run as a doublet) were detected in the two uninfected cell DNAs (lanes 1 and 8). Based upon hybridization with specific probes (data not shown), and comparison to the known EcoRI maps of the avian endogenous proviruses [60], the bands at 15 and 8.4 kb were identified as the proviral-cellular junction fragments of ev-1, the 11 and 8.6 kb bands as the junction fragments of ev-4, and the 4.2 kb band as the internal EcoRI fragment of both

ev-1 and ev-4. Thus, cells used for infection in this experiment contained the non-expressed endogenous proviruses ev-1 and ev-4 as defined by Astrin and others [2,3,53].

Clones 2-7 and 10-14 all contained the expected 2.5 and 5.0 kb internal fragments of BH-RSV. Only in clone 9 was a new pattern detected; the 5.0 kb fragment was absent, and instead a new band at 12 kb was detected. Since the 2.5 kb band in clone 9 was normal, the EcoRI site in the left-hand LTR and the internal site in gag were both present. This provirus appears to have lost the EcoRI site in the right-hand LTR and the 12 kb band should contain the remainder of the proviral 5.0 kb fragment linked to flanking cellular sequences downstream (rightward) of the provirus.

Figure 1.3. Clonal chicken cellular DNA digested with EcoRI and hybridized to viral probes. Samples (8 ug) of total cellular DNA were digested with EcoRI, subjected to agarose gel electrophoresis, and transferred to nitrocellulose paper as described in the text. (In lane 1 only 6 ug of DNA was used.) Lanes 1 and 8, cellular DNA from control uninfected CEF; lanes 2 through 7 and 9 through 13, independent BH-RSV alpha clones; lane 14, independent BH-RSV beta clone. Clones 2 through 7 were derived from the uninfected cells in lane 1, and clones 9 through 14 were derived from the uninfected cells in lane 8. The positions of the lambda DNA molecular weight markers run in a parallel lane are shown on the left. Endogenous proviral bands are indicated by "ev" on the right. All size indications are in kilobases. (A) Blot hybridized to RAV-2 $_{rep}$ probe. The internal BH-RSV fragments are indicated by their sizes on the right. Lane 1 is from a longer exposure. (B) Same blot as in (A) rehybridized to cDNA$_{5'}$. The BH-RSV proviral-cellular junction fragments are indicated by the arrowheads.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
23.72-
9.46-
6.67-
4.26-
2.25-
1.96-
-ev
—12
-ev
=ev
=ev
—5.0
-ev
—2.5
C
C
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
23.72-
9.46-
6.67-
4.26-
2.25-
1.96-
-ev
=ev
=ev
C
C

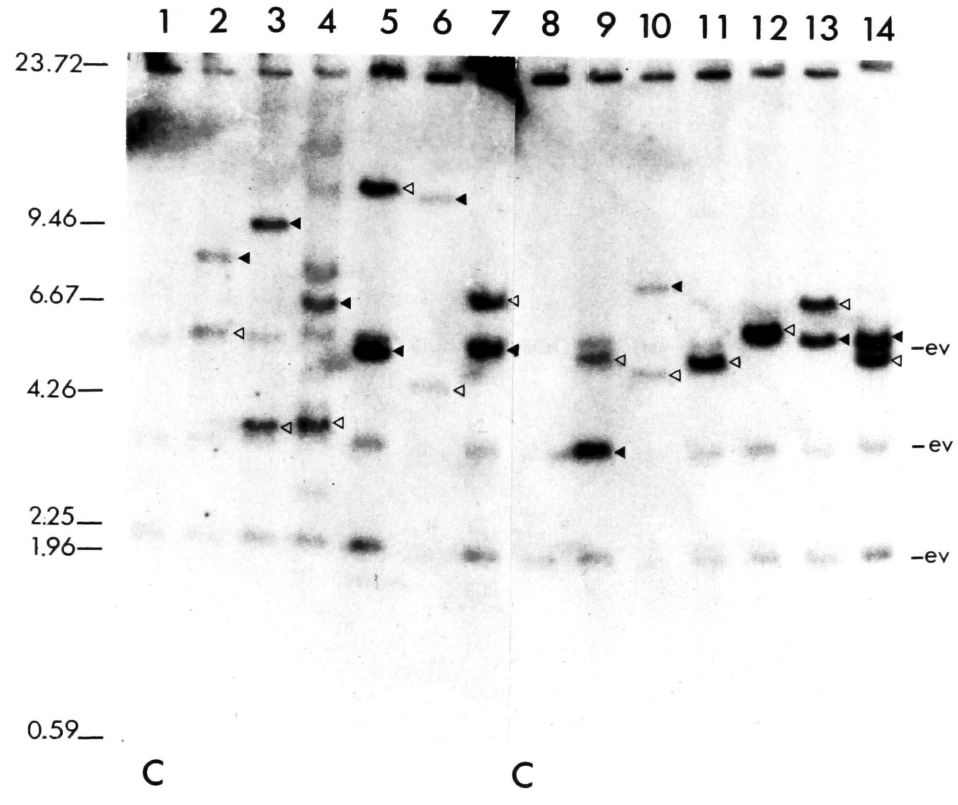
Number of proviruses and clonality of proviruses. DNA from clones 1-14 was digested with EcoRI and hybridized to $cDNA_{5'}$. $cDNA_{5'}$ will hybridize to the 2.5 kb internal fragment and to a provirus-cell junction fragment at the 3' end of the provirus. The number of new junction fragments in a sample is indicative of the number of proviruses present. The results of this analysis are shown in Fig. 1.3 (panel B). The bands in lanes 1 and 8, which contained DNA from uninfected cells, were the LTR-containing fragments of ev-1 and ev-4. The 2.5 kb internal fragment was detected in lanes 2-7, and 9-14. One unique junction fragment was detected in each sample for lanes 2-7, 10, and 12-14. The 12 kb junction fragment detected in clone 9 with $RAV\text{-}2_{rep}$ probe (Fig. 1.3, panel A) was not detected with $cDNA_{5'}$; the defect in this provirus must also affect the 5' sequences of the right-hand LTR. No junction fragment could be detected in clone 11, probably because the fragment was too small. Fragments smaller than 0.6 kb would run off this gel. Experiments with other enzymes did demonstrate the presence of junction fragments in clone 11 (see below). The junction fragment of clone 13 is difficult to see due to a partial "dead spot" in the nitrocellulose at that location; on longer exposure the indicated junction band became much more evident. To verify that the junction fragments in all the samples were truly unique, similar analyses were performed with other enzymes.

DNA from the 14 samples was subjected to digestion with HindIII, SacI, and KpnI, all of which cut BH-RSV DNA at only

one site (Fig. 1.2). DNA was hybridized to a probe specific for the LTR. Since the homology between the U_3 region of the LTRs of exogenous and endogenous viruses is limited [16], this probe shows preferential hybridization to exogenous proviral sequences.

As can be seen in Fig. 1.4, the enzyme HindIII generated a unique set of junction fragments in each sample. Analysis with SacI and KpnI gave similar results (data not shown). Occasionally, only one of the two junction fragments in a sample was detected when a particular enzyme was used (for example, in Fig. 1.4, lanes 11 and 12). This would be expected in cases where the second junction fragment is too large (or in the case of SacI fragments, too small) to be resolved on the particular gel. However, each sample which failed to reveal both junction fragments when cleaved with one enzyme, did reveal two junction fragments with the two other enzymes tested. Clone 9, which was found to be missing the EcoRI site as well as most of the 5' sequences of the right-hand LTR (Fig. 1.3), apparently retained most of the 3' information in that LTR. We detected both right and left-hand junction fragments in clone 9 when LTR probe was used on HindIII, SacI, and KpnI digestions (Fig. 1.4; data not shown). If clone 9 has a deletion it is likely that only the last 150 bp of the right-hand LTR are missing from the provirus. However, it is possible that the deletion extended into the flanking cellular sequences.

Figure 1.4. Clonal Chicken cellular DNA digested with HindIII and hybridized to the LTR probe. The contents of the lanes, the markings on the right, left, and bottom, and the experimental details were all as described in the legend to Fig. 1.3, except that the restriction enzyme was HindIII and the probe was the LTR. The junction fragments which mapped to the right end of the BH-RSV provirus are indicated by solid arrowheads; the junction fragments which mapped to the left end of the BH-RSV provirus are indicated by open arrowheads. The order of the fragments was determined by rehybridization of this blot to other viral probes (data not shown). The extra fragments in lane 4 were products of partial digestion.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
23.72
9.46
6.67
4.26
2.25
1.96
0.59
-ev
-ev
-ev
C
C

The blots from the HindIII, SacI, and KpnI analyses were re-hybridized to probes for selected parts of the RSV genome (data not shown) so that the restriction fragments could be ordered for construction of the maps shown in Fig. 1.5.

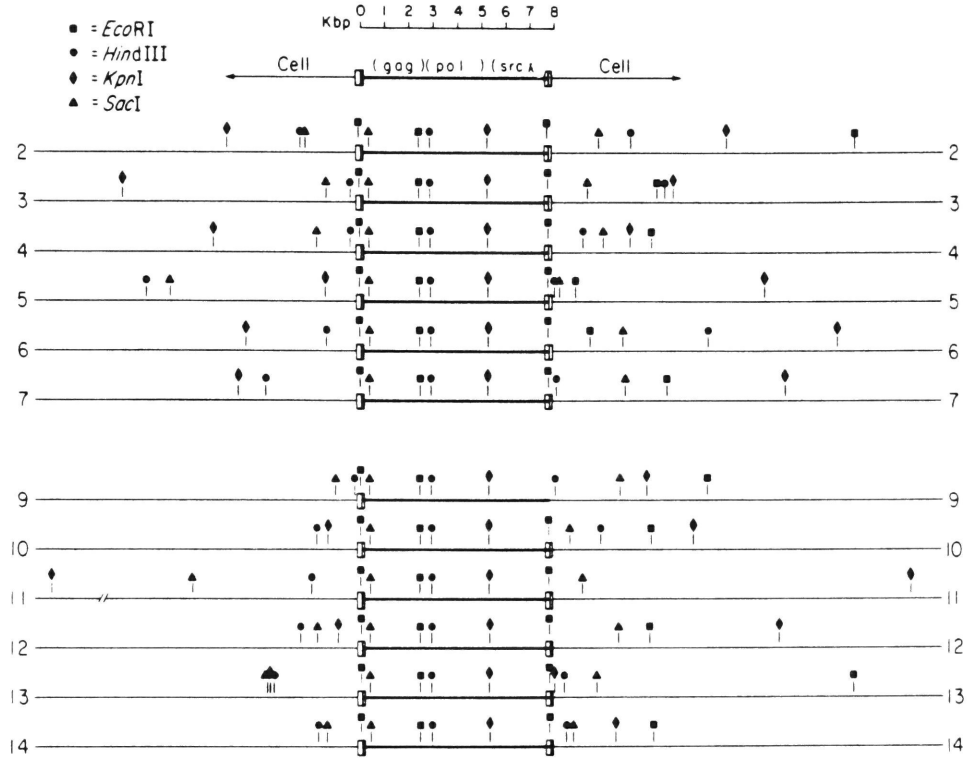


Figure 1.5. Restriction maps of the cellular integration sites of 12 independent BH-RSV-infected chicken cell clones. Cleavage sites for the restriction enzymes were determined by digestion of total cellular DNA and hybridization to specific viral probes, as described in the text. The scale and genetic map are shown at the top. The LTRs are depicted by boxes, as described in the legend to Fig. 1.2. The numbers at the ends of each line are the clone numbers, as described in the legend to Fig. 1.3. The break in the cellular DNA of clone 11 represents 6 kb. A right LTR is not depicted for the provirus in clone 9 since we determined that the provirus was missing viral sequences in that region (see text). There may be a deletion which spans the last 150 bp of proviral DNA; the deletion may also include an undetermined length of cellular DNA in the region immediately flanking the provirus of clone 9. Kbp, kilobase pairs.

DISCUSSION

Since the discovery of the site-specific integration mechanism of bacteriophage lambda into E. Coli [15,81], many investigators have examined the mechanism by which other elements integrate into their host cells. It has now been established that DNA tumor viruses such as SV40 integrate randomly with respect to their own genomes as well as the host DNA [11,76]. In avian and mammalian retroviruses, the integration event has been found to be extremely specific with respect to the viral genomes [18,20,38,61,62,111,122], with the LTR probably playing an important role in this specificity.

Isolation of RSV-Infected Cells Which Contained Single Proviruses

The studies reported here re-address the question of specificity using an avian retrovirus and its natural host cells under conditions which select for initial integration events. The virus employed (BH-RSV alpha) contains genetic defects in the pol and env genes. Initial infection was possible because both of these essential proteins were obtained from a complementing virus. Once infection was established under conditions where cells were infected by a single BH-RSV alpha(RAV-1) particle without a helper virus, the only viral genome present in the cells was that of the replication-defective BH-RSV alpha. Thus, multiple infection and virus spread were effectively prevented.

The results of Figs. 1.3B and 1.4 verified that clones of

infected chicken cells that harbored only a single provirus were obtained. This is in contrast to the results of others who were unable to obtain clonal populations of ASV-infected chicken cells by conventional methods [61,111]. Although the conditions we employed selected for infection by a single virus particle, the number of integration events which could be effected by a single particle was not under selective pressure. Our results therefore confirm the notion that single particles introduce single integration events. The appearance of multiple proviruses in a clone of infected cells, as has been reported by many investigators [7,38,61,111,129], can be primarily attributed to viral reinfection.

Expression of Viral Genes and Structure of Proviruses

The clones described were selected initially on the basis of transformed phenotype (src) and subsequently found to be positive for production of virus particles (gag). Thus, in each clone examined, the provirus was capable of supporting expression of the entire viral genome, regardless of the site of integration. Furthermore, as determined by restriction enzyme mapping, the cloning procedure did not cause the appearance of any grossly aberrant proviruses. Clone 9 was found to have a defect in its right-hand LTR. The transformed phenotype of clone 9 was indistinguishable from that of the other 11 clones, as were the levels of intracellular and virion-associated viral structural proteins (data not shown). In addition, transforming virus was successfully rescued from a plate of clone 9 cells superinfected with helper virus. It seems the defect in

this provirus did not affect any viral functions. Even if the signals for termination and polyadenylation of viral RNA transcripts were affected, there is evidence to suggest that viral RNA could be synthesized using cellular signals [151].

Lack of Common Integration Sites

Using four restriction enzymes, we found no similarities among the cellular integration sites of the 12 independent clones studied (Fig. 1.5). In addition there was no evidence of more than one provirus integrating into the same site in opposite orientations. Enzymes were found which cleaved at or very near the integration site in some but not all clones (Fig. 1.5). Thus, we conclude that the base sequence 100-500 nucleotides around the integration site differed among the clones. However these, as other similar studies, cannot eliminate the possibilities that 1) very short sequence similarities exist at or near the various possible integration sites, or 2) the provirus recognizes specific cellular sequences but integrates distal to such sites. Since the clones that were studied all survived the integration event, it was impossible to determine the number, if any, of "forbidden" sites for integration (i.e., into vital cellular genes). Within the limits mentioned, we detected no apparent preferred or specific acceptor site(s) for ASV. This finding supports the conclusion of others:

No preferential integration has been found in mouse cells infected with murine leukemia virus [4,129] or mouse mammary tumor virus [18,39]. In the avian system, RAV-0 [64], avian myeloblastosis associated virus-2 [7] and reticuloendotheliosis

virus [74,122] were found to be non-specifically integrated into chicken embryo fibroblasts. With Rous sarcoma virus, similar results were obtained in infected rat [19,62], duck [38], and quail cells [111]. This report and that of Hughes et al. [61] examine integration into chicken cells (the natural host).

The results presented in this report confirm and extend the findings of Hughes et al. Under conditions which select for initial integration events, Rous sarcoma virus integrated into chicken DNA at no preferred sites. In addition, expression of the proviral genes was shown to be compatible with proviral integration at multiple sites.

Recently, DNA fragments containing proviruses and the flanking host DNA have been molecularly cloned and examined. DNA sequence analyses of proviruses of several avian and mammalian retroviruses have revealed the consistent presence of a 4-6 bp duplication of host DNA at the site of integration [27,56,63,86,121,124]. There does not seem to be any specificity with regard to the sequence of the integration site (i.e., the repeated nucleotides), although the number of nucleotides which are duplicated seems to be characteristic of the retrovirus species and may reflect the involvement of a virally encoded function (possibly p19 or reverse transcriptase) in the integration process.

Keshet and Temin proposed that integration can occur at multiple sites in the genome, but biological expression of the proviral genes can only occur via integration at a "limited"

number of sites [74]. We found twelve expressed proviruses integrated at twelve different sites. Thus, this "limited" number, if it exists, must be significantly larger than twelve.

Oncogenesis by avian leukosis virus involves a "promoter-insertion" mechanism [97]. Although avian leukosis virus can express all its replicative functions via random integration, Neel et al. found that in avian leukosis virus-induced tumors, the proviruses were all integrated at a specific site. In light of their results, it seems likely that viral integration at specific sites would be found only in cases where there is selection for expression of a function which is not encoded on the viral genome (such as tumor formation by avian leukosis virus). Transformation by avian sarcoma virus would not be in this category.

Chapter 2

Genetic structure of BH-RSV: The *pol*-*src* junction and the *src*-U_3 region

INTRODUCTION

Both BH-RSV alpha and beta have been used extensively in genetic analyses of RSV. The *pol* and *env* defects have been well characterized biologically; to complete the biochemical characterization of the defects in the BH-RSV strains, DNA fragments containing the defective regions were molecularly cloned and analyzed.

In this study, I examined the sequence of the *pol*-*src* region of BH-RSV to determine whether residual *env* coding information was present on the BH-RSV genome. The mechanism of generation of this deletion is not known, and the regions flanking the deletion were examined for the presence of homologous sequences which may facilitate the process of deletion.

Recent DNA sequencing of the *src* genes of two other strains of RSV, SR- and PR-RSV, revealed the presence of direct repeats and other features in the non-coding regions flanking the 5' and 3' sides of *src* [117,135,136]. The sequence of the homologous region in BH-RSV was determined in an attempt to gain insight into the recombination events which resulted in transduction of the c-*src* gene into a retrovirus.

The sequence of BH-RSV was also examined in an effort to obtain information regarding the genesis of BH-RSV. Because of uncertainties in the passage history of the RSV strains (see

introductory chapter), it is not actually known whether BH-RSV was a deletion mutant of a non-defective RSV or an independent recombinant between a helper virus and c-*src*.

The results indicated that essentially the entire *env* and 5' flanking region of *src* were absent from BH-RSV. Comparisons between BH-RSV sequences and other retrovirus sequences, revealed the presence of two regions of highly conserved non-coding sequences. The origin of BH-RSV could not be determined since the sequence did not provide strong evidence for either the *env* deletion or independent recombinant model.

RESULTS

Analysis of Molecular Clones of BH-RSV

Molecular Cloning of 5 kb pol-src fragment of BH-RSV alpha and beta. The 5 kb EcoRI fragment of BH-RSV alpha and beta was cloned from the infected cells which were used in the integration study (chapter 1). DNA from BH-RSV alpha chicken cell clones 2-7 and 10-13, and DNA from BH-RSV beta rat 3Y-1 cells were completely digested with EcoRI, fractionated on a sucrose gradient, and DNA fractions containing the 5 kb viral pol-src fragment were cloned into the EcoRI site of the phage vector, lambda gt WES. For both BH-RSV alpha and BH-RSV beta, a lambda clone containing the 5 kb EcoRI pol-src fragment was isolated and purified. In order to increase the ratio of insert DNA to vector DNA, the 5 kb viral fragment was subcloned into the EcoRI site of the plasmid pBR322. Plasmid clones containing the 5 kb fragment from either BH-RSV alpha or beta were isolated; one clone of each, refered to as pBH-alpha or pBH-beta, was chosen for further analysis. The structure of these plasmid clones is shown in Figure 2.1.

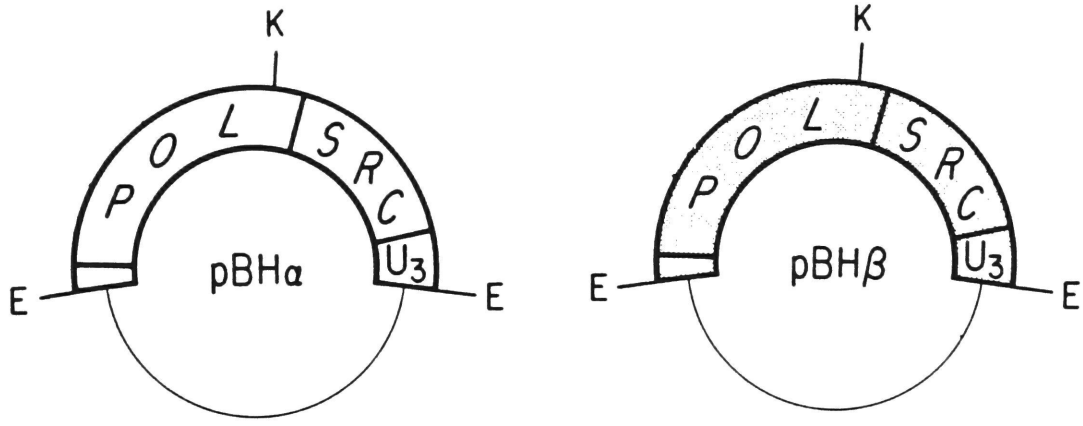


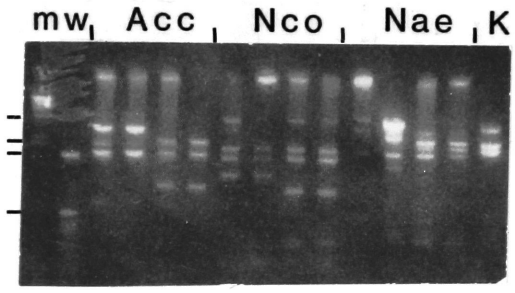
Figure 2.1. Structure of BH-RSV molecular clones. The upper portion of the circles represent the 5 kb EcoRI viral insert fragments. The lower portion represents pBR322 sequences (4.3 kb). Restriction enzyme sites which are indicated are: E, EcoRI; K, KpnI.

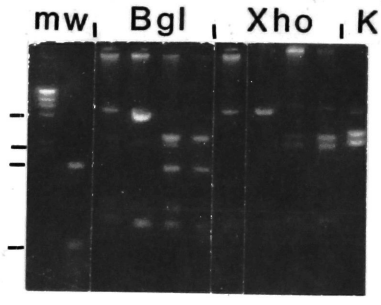
Restriction enzyme cleavage analysis of pBH-alpha and pBH-beta. Based on the nucleotide sequence of the entire genome of PR-RSV [117] and the env-src EcoRI fragment of SR-RSV [134], restriction enzymes with cleavage sites in env were selected for analysis of the BH-RSV genome. Figure 2.2 shows some of the restriction enzyme cleavage data for pBH-alpha and pBH-beta.

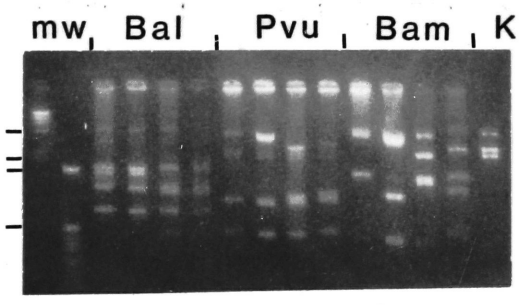
To orient the DNA fragments which were obtained by digestion with the various restriction enzymes, secondary digestion with the enzyme KpnI was performed. The single KpnI site is located approximately 200 bases before the stop codon of pol in PR-RSV, essentially dividing the genome into a gag-pol half and an env-src half. KpnI digestion of the 5 kb pol-src EcoRI fragment of BH-RSV yielded two fragments of approximately 2.7 and 2.4 kb which represent the left and right halves, respectively, of the 5 kb fragment (lane K, see also Figs. 1.1 and 1.2).

The order and identity of the restriction fragments shown in the gels in Fig. 2.2 was determined by comparison of single and double digests, comparison of the sizes of the BH-RSV fragments to those of SR- and PR-RSV, and hybridization of a Southern blot of the gel with src specific probe (not shown).

Figure 2.2. Restriction enzyme cleavage analysis of pBH-alpha and pBH-beta. The 5 kb EcoRI viral insert fragments were digested with enzymes as described below and subjected to electrophoresis through 1.5% agarose in a "mini gel" apparatus. Gels were stained with ethidium bromide. In lanes marked mw, the first lane was a HindIII digest of lambda DNA and the second lane was a HinfI digest of pBR322 DNA. The markings on the left indicate the positions of the 4.3, 2.0, 1.6, and 0.5 kb markers. K, KpnI digest of pBH-beta insert. Lanes marked with names of enzymes: in each set of 4 lanes, the first and third lanes were pBH-alpha insert DNAs and the second and fourth lanes were pBH-beta insert DNAs. The first and second lanes were single digests with the indicated enzyme and the third and fourth lanes were double digests with the indicated enzyme and KpnI. The faint band at 4.3 kb in many lanes was pBR322 DNA. Enzymes used were AccI, NcoI, and NaeI (upper left); BalI, PvuII, and BamHI (lower left); and BglI and XhoI (right).


mw
Acc
Nco
Nae
K


mw
Bgl
Xho
K


mw
Bal
Pvu
Bam
K

The individual restriction enzyme fragments which were cleaved by secondary digestion with KpnI were identified as fragments which span the pol-src junction. BH-RSV fragments which were contained wholly in pol or src were found to be the same size as the corresponding fragments of SR- or PR-RSV, with the exception of: 1) An XhoI site, not found in PR- or SR-RSV, was present in the pol gene of BH-RSV, approximately 800 bp from the 5' end of the 5 kb insert of pBH-alpha and pBH-beta (note the 800 bp band in XhoI digests shown in Figure 2.2) 2) A PvuII site in pol, present in PR-RSV, was not found in either pBH-alpha or pBH-beta. 3) A second BamHI site, mapping near the 3' end of the PR-RSV pol gene, was not present in pBH-alpha or pBH-beta. This site is also not present in SR-RSV [26]. However, an additional BamHI site mapping near the 5' end of the pol gene was found in pBH-beta. This site was not present in pBH-alpha (note the different patterns for BamHI digestion of pBH-alpha and beta), SR-RSV, or PR-RSV.

Except for BamHI digests, restriction fragments of both pBH-alpha and pBH-beta comigrated indicating that the additional pol defect in BH-RSV alpha was probably not due to a large deletion or major sequence rearrangement. (The apparent difference between the digestion patterns of pBH-alpha and beta with the enzyme NaeI was not consistently observed and was believed to be an artifact in this particular reaction).

The results obtained from the restriction enzyme cleavage analysis are summarized in Figure 2.3. All the restriction enzyme sites which map in pol or src (with the exception of the

few discussed above) were present in BH-RSV. All the restriction enzyme sites which map in either *env* or the intercistronic region between *env* and *src* were absent. The *Nco*I site, shown in the figure to map at the border between the intercistronic region and *src*, was present in BH-RSV. This cleavage site includes the ATG initiation codon (recognition site for *Nco*I: CCATGG) of the PR- and SR-RSV *src* genes; the presence of the *Nco*I site in BH-RSV suggests that the *src* coding region is intact in BH-RSV.

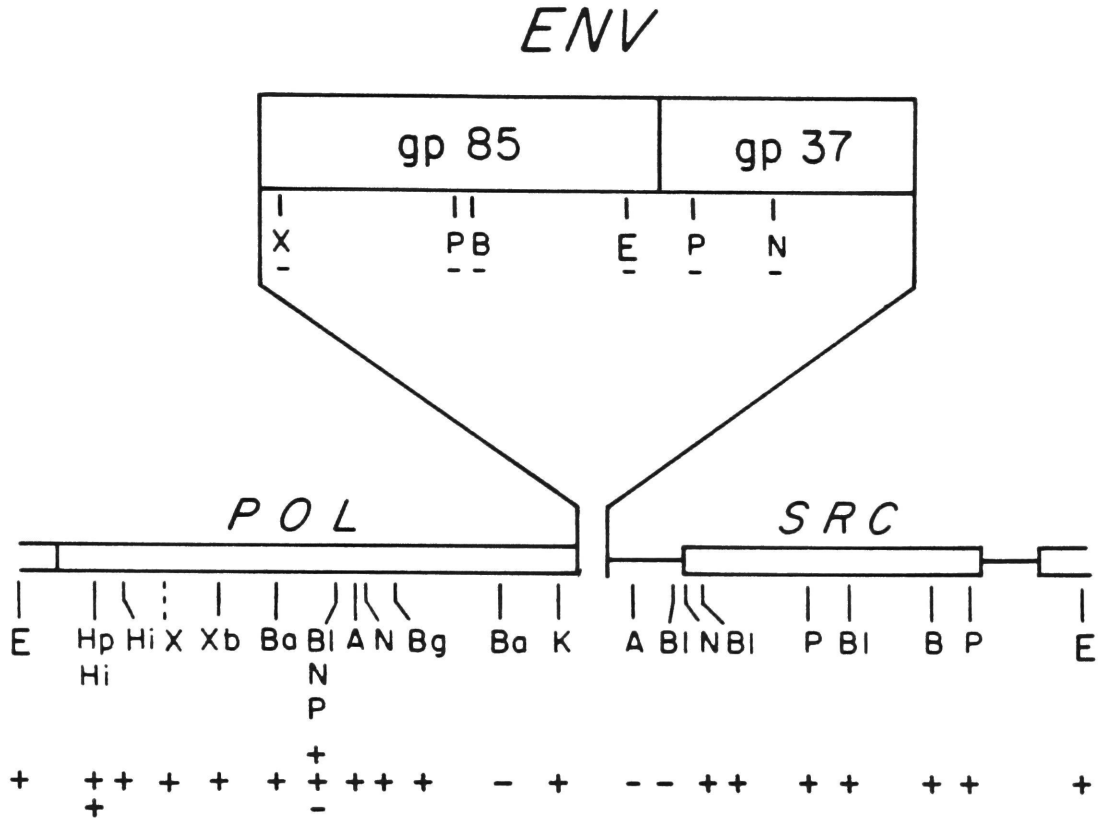
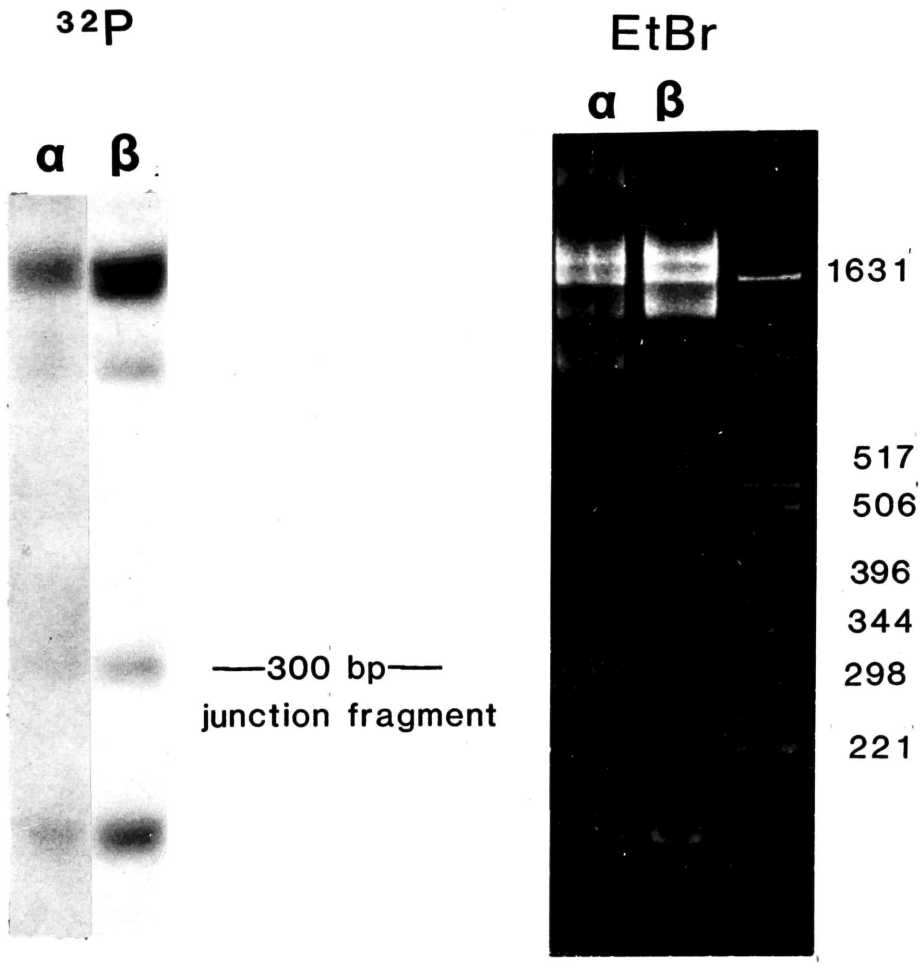


Figure 2.3. Restriction enzyme cleavage map of BH-RSV compared to non-defective RSV. Positions of restriction enzyme cleavage sites on the RSV genome between the EcoRI site in gag and the EcoRI site in U_3 are indicated. Enzyme sites in pol and gp85 were based on the PR-RSV sequence [117] and sites in gp37 through U_3 were based on the SR-RSV sequence [134]. The env gene was drawn to a larger scale. A (+) or (-) sign below a position indicates whether the site was present or absent in BH-RSV; the map refers to both BH-RSV alpha and beta. Dotted line marking an enzyme site indicates a site which was present in BH-RSV but not present in PR-RSV. An extra BamHI site which was present in BH-RSV beta only (see text) is not shown; it mapped between X and Xb in the 5' portion of pol. Enzymes were E, EcoRI; Hp, HpaI; Hi, HindIII; X, XhoI; Xb, XbaI; Ba, BamHI; Bl, BalI; N, NcoI; P, PvuII; A, AccI; Bg, BglII; K, KpnI; B, BglI.

Sizing of a pol-src junction fragment. To further examine the extent of the missing env sequences in BH-RSV alpha and beta, the size of the pol-src junction fragment, bounded by the KpnI site in pol and the NcoI site at the 5' end of src, was determined. As shown in Figure 2.4, the KpnI-NcoI fragment in BH-RSV was approximately 300 bp. The homologous fragment of PR- or SR-RSV would be approximately 2.2 kb. If the 3' end of the BH-RSV pol gene is similar to that of PR-RSV, it would be expected that following the KpnI site would be 200 bp of pol, thus allowing for no more that 100 bp of env or intercistronic sequences in BH-RSV.

As can be seen in Figure 2.4, the KpnI-NcoI fragment of BH-RSV alpha and BH-RSV beta comigrate, suggesting that the pol defect in BH-RSV alpha is not due to an extension of an env deletion into the pol gene.

Figure 2.4. NcoI-KpnI double digests. 5 kb insert of pBH-alpha or pBH-beta was digested with NcoI followed by: (left panel) 5' end-labeling with ^{32}P and secondary digestion with KpnI, or (right panel) secondary digestion with KpnI. Electrophoresis was performed on 5% polyacrylamide gels; molecular weight markers shown in rightmost lane were HinfI digests of pBR322 DNA. Panel on left was an autoradiogram, panel on right was an ethidium bromide-stained gel.


32P
α β
—300 bp—
junction fragment
EtBr
α β
1631
517
506
396
344
298
221

DNA Sequence Analysis of BH-RSV

Sequence of the pol-src junction in Bryan RSV. The 300 bp KpnI- NcoI fragment was partially sequenced by the method of Maxam and Gilbert [92] according to the strategy depicted in Figure 2.5. The NcoI 5' end was labeled with ^{32}P in a kinase reaction and sequencing in the reverse direction (non-coding strand) from the initiation codon of src revealed that there were only 97 bp between the end of pol and the beginning of src in BH-RSV (Figure 2.5). The 91 nucleotides preceding src in BH-RSV were found to be essentially identical to the 91 nucleotides preceding src in PR- and SR-RSV, in recombinant rASV 1441 [136], and in c-src [135], the cellular homolog of the viral src gene. This 91 bp region contains the putative splice acceptor site (SA) for src mRNA, mapped to position -74 relative to src [117,136].

Immediately preceding this 91 bp region (SA region) in BH-RSV was the sequence identified as the end of the pol gene, including the stop codon TAA and 6 nucleotides which follow pol in PR-RSV. The pol-src junction sequence was confirmed by sequencing using a DdeI site as the labeled end; partial sequencing of the 3' end of the BH-RSV pol gene confirmed that the sequence was essentially identical to that of PR-RSV.

A helper virus-related sequence, which is present between env and src and repeated between src and the LTR in both SR- and PR-RSV, was not present in the region 5' of src in BH-RSV.

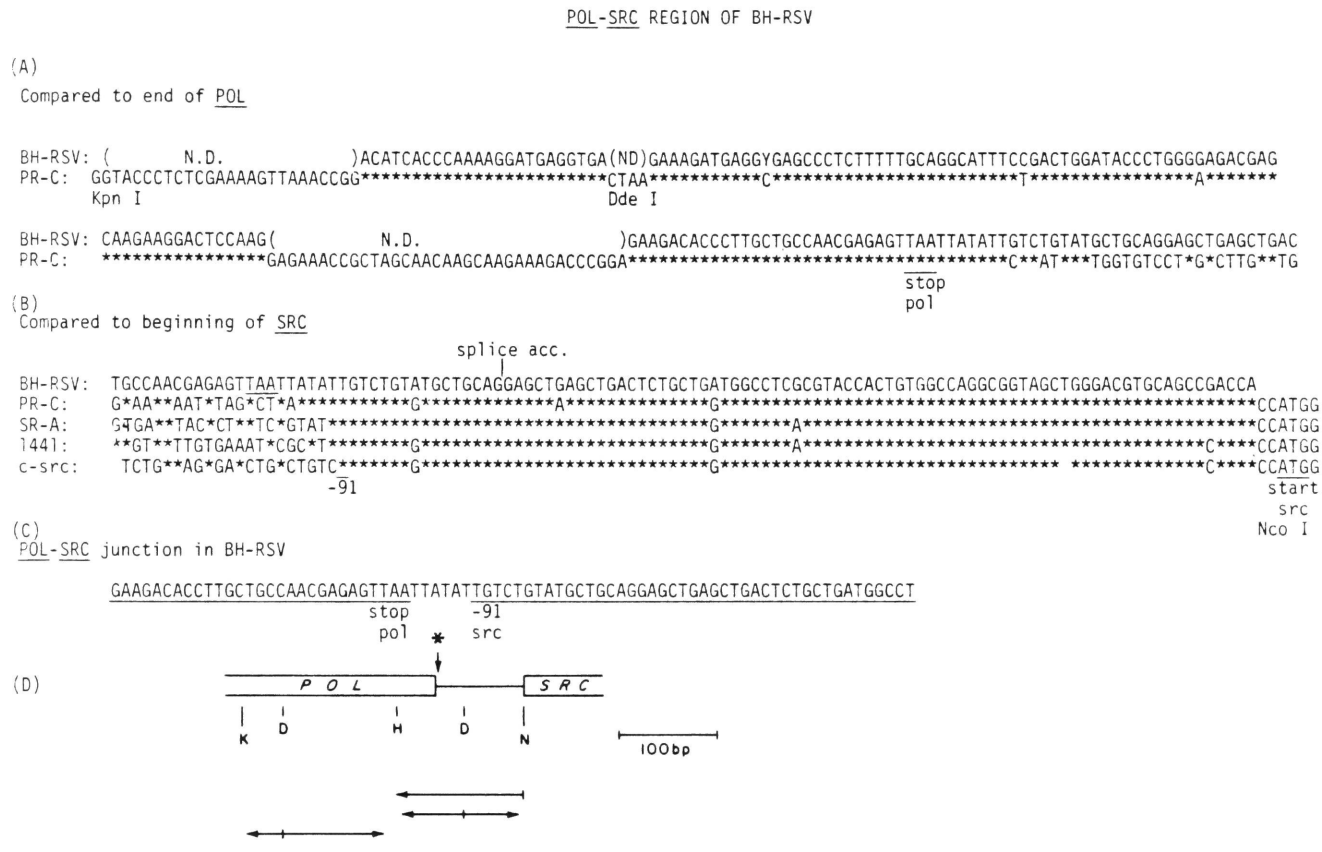


Figure 2.5. DNA sequence of 300 bp NcoI-KpnI fragment of BH-RSV. (A) BH-RSV sequence from KpnI site to end of pol gene compared to sequence of homologous region of PR-RSV [117]. (B) BH-RSV sequence from the end of pol to the NcoI site, compared to sequence upstream from src in PR-RSV [117], SR-RSV [134], rASV 1441 [136], and c-src [135]. Termination codon of pol in BH-RSV is underlined. In (A) and (B), * indicates the same nucleotide as BH-RSV and (N.D.) indicates sequences which were not determined. (C) Highlight of the pol-src junction in BH-RSV. (D) Schematic showing region sequenced and strategy used. Asterisk with arrow indicates position of pol-src junction as determined by DNA sequencing. Enzymes: K, KpnI; D, DdeI; H, HpaI; N, NcoI.

3' end of Bryan RSV. Preliminary restriction enzyme mapping analysis indicated that the last 500 bp of pBH-beta were similar to the corresponding region of SR-RSV except for a short region immediately following the src gene. In this region, BH-RSV did not contain the restriction enzyme cleavage sites present in the corresponding region of SR-RSV (Figure 2.6). Hybridization experiments indicated that neither the sequence termed "F3" (found on the 3' end of SR-RSV) nor "F2" (found on the 3' end in PR-RSV) [135] were present in BH-RSV (data not shown).

The 3' end of the BH-RSV beta, from the last 70 bp of src to the EcoRI site in the U_3 region, was sequenced according to the strategy depicted in Figure 2.6. The results (Figure 2.7) show an overall similarity between the BH-RSV sequence and the corresponding SR-RSV sequence, with one major exception. Immediately following the stop codon of src, the next 118 nucleotides of BH-RSV and SR-RSV were completely different. Except for a few single base changes, and a 12 bp repeat present in BH- and not in SR-RSV, the remainder of the sequence was the same in both strains.

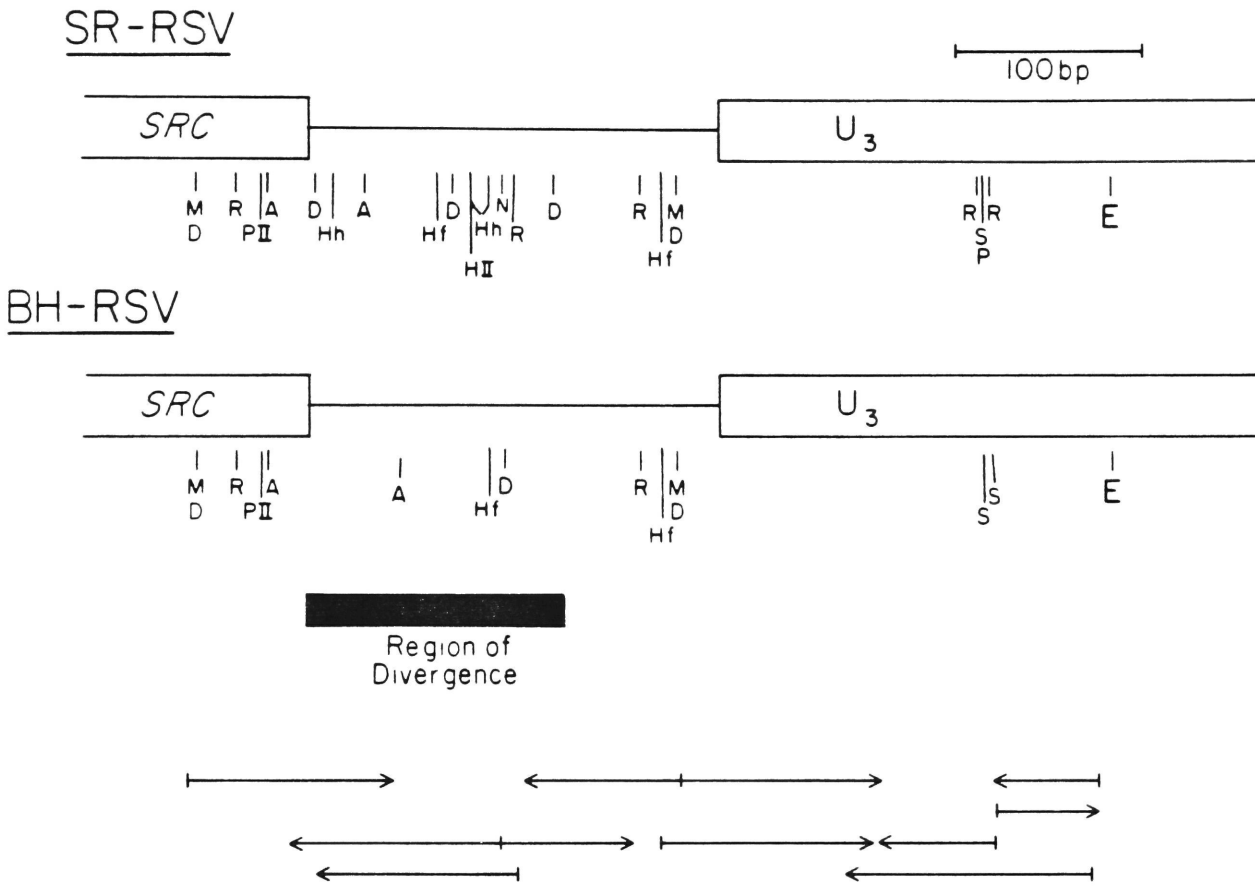


Figure 2.6. Comparison of the 3' end of BH-RSV and SR-RSV. Positions of restriction enzyme cleavage sites in the region between the end of src and the U_3 region are shown. SR-RSV sites are based on DNA sequence in ref. 134. BH-RSV sites based on restriction mapping analysis of pBH-beta. Strategy for sequencing this region of BH-RSV is shown at the bottom of the figure. Enzymes: M, MstII; D, DdeI; R, RsaI; PII, PvuII; A, AluI; Hh, HhaI; Hf, HinfI; HII, HaeII; N, NruI; S, Sau3A; P, PvuI; E, EcoRI.

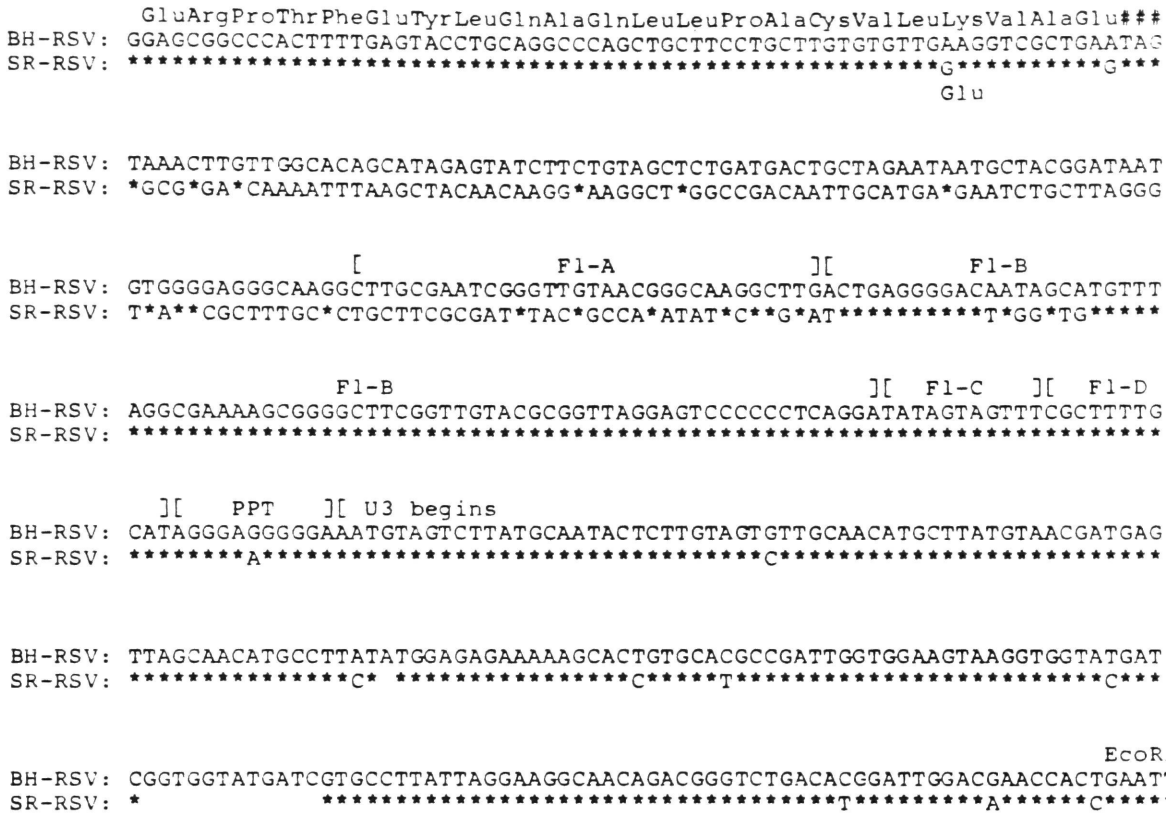
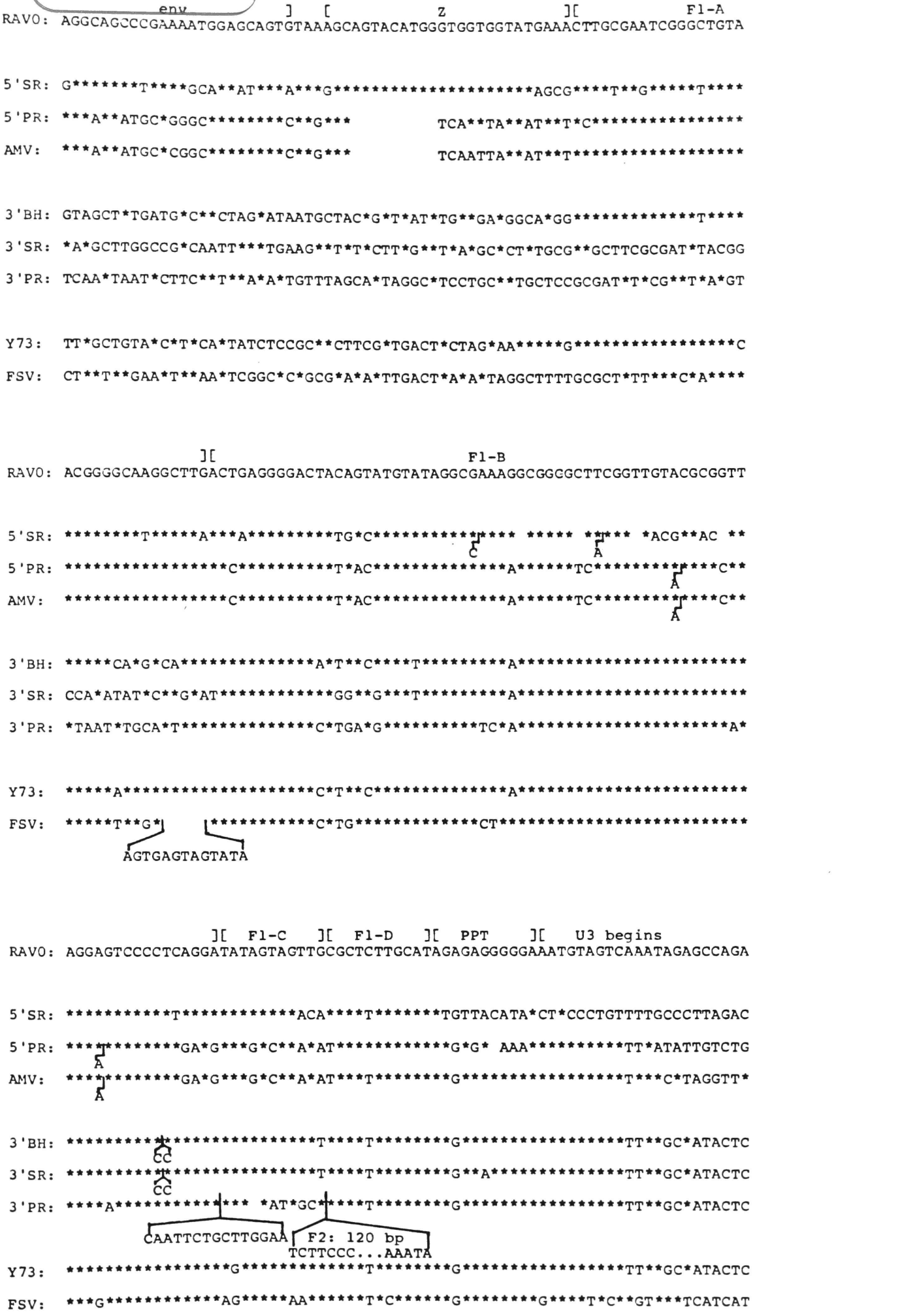


Figure 2.7. DNA sequence of the 3' end of BH-RSV compared to homologous region of SR-RSV. Amino acids shown represent carboxy terminal residues of src. ###, termination codon of src. F1A-F1D, see text for explanation. PPT, polypurine tract. *, same nucleotide as BH-RSV. Blank space indicates nucleotide not present in SR-RSV.

Conservation of a helper virus-related sequence in avian retroviruses. One copy of "F1", the sequence present as a direct repeat on either side of src in SR- and PR-RSV [117,134], was found on the 3' side of src in BH-RSV (Fig. 2.7). Comparison of the two copies of F1 in SR- and PR-RSV indicated that the sequence seemed to be composed of discrete blocks of conserved sequences which I termed F1 A-D.

Since the F1 sequence is also present between env and U_3 in RAV-0 [59], F1 is believed to be of helper virus origin. Examination of the DNA sequence of several avian retroviruses revealed the remarkable fact that the F1 sequence is present immediately upstream from U_3 in every virus for which the sequence in this region is known [77,78,110,117,120,134]. Figure 2.8 shows the F1 sequence present in RAV-0, PR-RSV, SR-RSV, AMV, FSV, Y73 and BH-RSV. In addition, F1 is also present in ev1, ev2, and RAV-2 [A.M. Skalka, et al personal communication]. F1 is not present in murine retroviruses [123].

Figure 2.8. Comparison of the F1 sequence of RAV-0 with that of several avian transforming retroviruses. RAV0: RAV-0; sequence shown is 3' end of *env* through U_3 [59]. Z, sequence between the termination codon of *env* and start of F1 in RAV-0. Other regions are the same as described in legend to Figs. 2.7 and 2.8. 5'SR and 5'PR: 5' side of *src* in SR- and PR-RSV respectively. Sequence shown is the 3' end of *env* through the *env*-*src* intercistronic region [117,134]. 3'BH, 3'SR, and 3'PR: 3' side of *src* in BH-, SR-, and PR-RSV respectively. Sequence shown is downstream of *src* through U_3 [Fig. 2.7 and 117,134]. In AMV, Y73, and FSV the sequence shown is downstream of the *onc* sequence through U_3 [77,78,110, 120]. *, same nucleotide as RAV-0; blank space indicates a nucleotide which was not present. Inserted nucleotides are indicated.


env
] [z][F1-A
RAV0: AGGCAGCCCGAAAATGGAGCAGTGTAAAGCAGTACATGGGTGGTGGTATGAAACTTGCGAATCGGGCTGTA
5'SR: G*******T****GCA**AT***A***G**********AGCG****T**G*****T****
5'PR: ***A**ATGC*GGGC********C**G*** TCA**TA**AT**T*C****************
AMV: ***A**ATGC*CGGC********C**G*** TCAATTA**AT**T******************
3'BH: GTAGCT*TGATG*C**CTAG*ATAATGCTAC*G*T*AT*TG**GA*GGCA*GG*************T****
3'SR: *A*GCTTGGCCG*CAATT***TGAAG**T*T*CTT*G**T*A*GC*CT*TGCG**GCTTCGCGAT*TACGG
3'PR: TCAA*TAAT*CTTC**T**A*A*TGTTTAGCA*TAGGC*TCCTGC**TGCTCCGCGAT*T*CG**T*A*GT
Y73: TT*GCTGTA*C*T*CA*TATCTCCGC**CTTCG*TGACT*CTAG*AA*****G****************C
FSV: CT**T**GAA*T**AA*TCGGC*C*GCG*A*A*TTGACT*A*A*TAGGCTTTTGCGCT*TT***C*A****
][F1-B
RAV0: ACGGGGCAAGGCTTGACTGAGGGGACTACAGTATGTATAGGCGAAAGGCGGGGCTTCGGTTGTACGCGGTT
5'SR: ********T*****A***A*********TG*C*********** ***** **** *ACG**AC **
C A
5'PR: ****************C**********T*AC**********A******TC**********C**
A
AMV: ****************C**********T*AC**********A******TC**********C**
A
3'BH: *****CA*G*CA**********A*T**C****T*********A************************
3'SR: CCA*ATAT*C**G*AT**********GG**G***T*********A************************
3'PR: *TAAT*TGCA*T**********C*TGA*G**********TC*A**********A*
Y73: *****A*********************C*T**C**********A************************
FSV: *****T**G* |**********C*TG**********CT**************************
AGTGAGTAGTATA
][F1-C][F1-D][PPT][U3 begins
RAV0: AGGAGTCCCCTCAGGATATAGTAGTTGCGCTCTTGCATAGAGAGGGGGAAATGTAGTCAAATAGAGCCAGA
5'SR: **********T***********ACA****T*******TGTTACATA*CT*CCCTGTTTTGCCCTTAGAC
5'PR: ************GA*G***G*C**A*AT***********G*G* AAA**********TT*ATATTGTCTG
A
AMV: ************GA*G***G*C**A*AT***T********G*****************T***C*TAGGTT*
A
3'BH: **********************T****T********G*****************TT**GC*ATACTC
CC
3'SR: **********************T****T********G**A**************TT**GC*ATACTC
CC
3'PR: ****A********** *AT*GC*****T********G*****************TT**GC*ATACTC
CAATTCTGCTTGGAA F2: 120 bp
TCTTCCC...AAATA
Y73: ****************G***********T********G*****************TT**GC*ATACTC
FSV: ***G***********AG*****AA******T*C******G********G****T*C**GT***TCATCAT

Comparison of the sequences flanking src in various RSVs. Figure 2.9 summarizes the relationship between the flanking regions of BH-RSV and those of two other RSVs and a helper virus. In contrast to the other RSVs, BH-RSV does not have any flanking sequences 5' of the src gene. The 91 bp SA region is directly linked to the end of pol. On the 3' side of src, BH-RSV has one complete copy of the RAV-0 F1 sequence. The first copy of the SR- and PR-RSV F1 sequence (on the 5' side of src) seems to be complete; however the second copy of F1 (on the 3' side of src) in these sarcoma viruses lacks the F1-A region. In PR-, SR-, and BH-RSV, the ~120 nucleotides immediately following src are unrelated and seem to be unique to the individual strains.

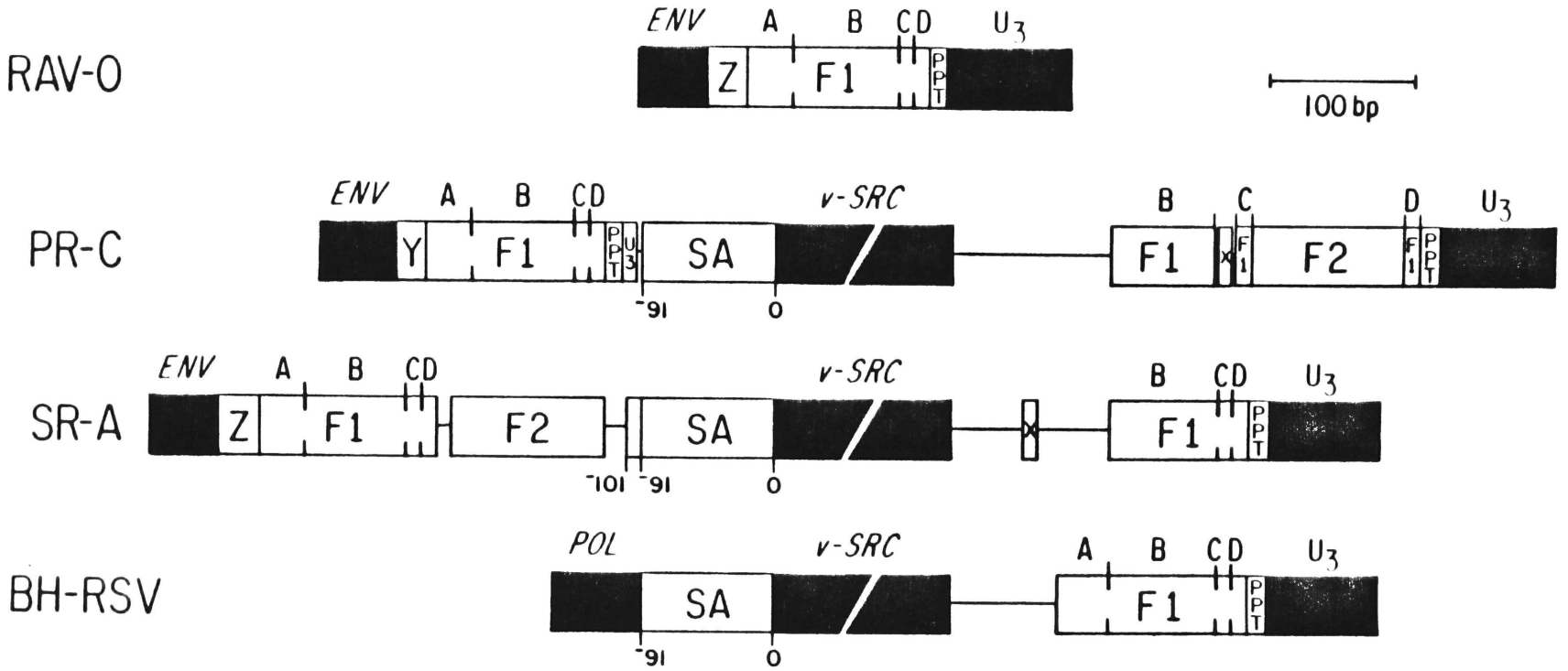


Figure 2.9. Arrangement of non-coding sequences flanking src and comparison to sequences of helper virus. PR-C, PR-RSV subgoup C; SR-A, SR-RSV subgroup A. Z, F1, and PPT were described in legend to Figs. 2.7 and 2.8. Y, sequence between env and F1 in PR-RSV; SA, 91 bp sequence preceding src coding sequences (in SR-RSV -101 to -91 is a duplication of -91 to -81); F2, a 120 bp sequence found in PR-RSV and SR-RSV. X, a 10 bp sequence found in different positions on the 3' side of src in PR- and SR-RSV.

DISCUSSION

Env Deletion in BH-RSV.

On the basis of DNA sequence data (Fig. 2.5), I estimate the deletion in BH-RSV to be approximately 1.9 kb. The exact number of nucleotides which are deleted can not be determined because of the variation in the size of this region in different non-defective RSVs (Fig. 2.9). The sequence data are in good agreement with previous size estimates based on restriction enzyme mapping analysis (i.e., 2 kb) [118]; however the deletion is slightly more extensive than the estimate reported on the basis of oligonucleotide fingerprinting analysis (i.e., 1.5 kb) [30].

Sequencing revealed the gp85 and gp37 coding sequences to be completely absent from BH-RSV. Only the portion of the *env* signal sequence which overlaps with the 3' end of *pol* (in another reading frame) [see 117] as well as 6 bp which follow the *pol* terminator were present in BH-RSV. Since the putative splice acceptor site for *env* mRNA maps within the 3' end of *pol*, BH-RSV may, in fact, synthesize 2 species of 21S *src*-containing mRNA which differ by ~135 nucleotides. (The larger mRNA species would not be expected to direct the synthesis of a *src*-related protein since it would be translated in a different reading frame than *src*; this reading frame is also interupted by several termination codons in the *pol*-*src* junction). In addition to the absence of *env*, the F1 sequence or other flanking sequences were not present upstream from *src*. The region between *pol* and *src* in BH-RSV, therefore, does not share any

homology with ALV and this may explain the inability of BH-RSV to recombine with ALV to generate a non-defective RSV [70].

The sequence surrounding the pol-src junction of BH-RSV was examined for sequence information which may shed light on the mechanism of such a deletion. src gene deletions occur with high frequency in RSVs resulting in the formation of tdRSV mutants [70] and examination of the DNA sequence surrounding the src gene suggests a mechanism for this deletion. In both PR- and SR-RSV strains, the F1 sequence is repeated on the 5' and 3' sides of the src gene [17,134]; homologous recombination between the two F1's would result in loss of all sequences in between, including src. Uniformity in the size of many independently derived td isolates [31] as well as DNA sequence data [151] supports this hypothesis. (However, partial tds with smaller src deletions [73] were most probably generated by other mechanisms.)

Examination of the sequence surrounding the pol-src junction in BH-RSV and comparison to other available sequence data, did not reveal any obvious sequence homologies which would support the notion that a deletion occurred by homologous recombination. In PR-RSV the sequence immediately following pol (TTATATTCTC) shares 8 out of 10 bp with a sequence upstream from src (TAATATTGTC, -97 to -88 relative to PR-RSV src). A homologous recombination occurring within the 5 bp homology (ATATT) in these two regions would generate a the sequence of the pol-src junction. However, it is unlikely that such a short homology, 5 bp within an otherwise non-homologous sequence, could

account for the BH-RSV deletion. This would be equivalent to proposing that restriction fragments could be deleted by "homologous recombination" between cleavage sites, a phenomenon that has not been reported to occur.

Although the DNA sequence of the pol and env genes of SR-RSV is not available, comparison of the sequence at the end of PR-RSV pol with the sequence upstream from SR-RSV src revealed only a 2 bp homology with the BH-RSV pol-src junction. Therefore, on the basis of sequence data available at the present time, it appears unlikely that the BH-RSV gene structure is a result of deletion by homologous recombination. However, because of the limited amount of available DNA sequence data on pol and env, we are still unable to rule out this possibility.

One approach to determine whether there is a feature inherent in the sequence which renders the env region susceptible to complete deletion would be to examine the pol-src junction of other deletion mutants. SR-NY8 is an env-deletion mutant derived from SR-RSV [71]. Studies are currently in progress to determine the extent of the deletion in this strain. An identical or similar pol-src junction would suggest a common mechanism of deletion.

The 3' End of BH-RSV.

The sequence of the 3' end of BH-RSV failed to demonstrate a relationship between BH-RSV and either of the two non-defective viruses, PR- or SR-RSV (Figs 2.7 and 2.9). Although this may suggest that BH-RSV did not derive from either of these strains by deletion of env, these sequence data are in-

sufficient to conclude that BH-RSV is an independently derived defective sarcoma virus. Too few viruses have been sequenced thus far and general conclusions about the origins of the various strains cannot be drawn at this time.

Retroviruses have been observed to undergo recombination at a very high rate when mixtures of viruses are passaged or when virus is passaged through cells containing endogenous viral information [48,70]. Because the passage history of the RSV strains involved several decades of in vivo passage, it is very important to examine the sequences of endogenous proviruses and several helper viruses. Portions of the RSV genome which were not subjected to selective pressure may have been derived from endogenous or helper viruses by recombination. In fact, it has come to our attention that the BH-RSV sequence between src and the EcoRI site in U_3 is also found in RAV-2 [Bizub and Skalka, personal communication]. It is very interesting to note that RAV-2 was originally isolated as a helper virus in BH-RSV stocks [41]. The differences in the non-coding regions of BH-, PR-, and SR-RSV may therefore reflect the nature of passenger helper viruses more than the origins of the transforming viruses.

Conservation of a 91 bp Region Preceding src.

Takeya and Hanafusa reported that the sequence of the c-src exons is highly conserved in the v-src genes of SR-RSV and rASV 1441, and that the sequence conservation includes 91 bp of non-coding information immediately preceding c-src [134,135]. It was therefore proposed that position -91 relative to the

start of c-src represents the site at which recombination occurred between a helper virus and the c-src gene to generate the the transforming virus, RSV. Additionally, it was suggested that this same position was involved in the recombination events which resulted in the generation of rASV by recombination between tdRSV and c-src [134]. Remarkably, the pol-src junction in BH-RSV occurs at the very same position, -91 relative to src (Fig. 2.5). Examination of the sequence data reveals that BH-, PR-, and SR-RSV have essentially the same nucleotide sequence from position 0 to -91 relative to src; upstream from this point the three RSVs diverge from each other and from c-src.

Some implications of this finding include: 1) Conservation of this sequence would suggest a functional requirement for this entire region. Position -91 is approximately 12 bp upstream from a splice acceptor "consensus sequence" [83] which has been proposed to be the acceptor site for src mRNA [117,136]. Deletions extending into the consensus sequence and beyond may impair the the virus' ability to synthesize a functional src gene product. 2) If BH-RSV is an independent defective sarcoma virus, it would appear that the identical site in c-src could participate in recombination events with helper virus at (at least) two different sites on the helper virus genome.

In either case, it is not clear what structural feature or other property is inherent in the DNA sequence in the vicinity of -91 to make it such a "hot spot" for recombination and/or

deletion.

Conservation of the "F1" Sequence.

The striking conservation of this ~125 bp non-coding sequence in so many avian retroviruses would suggest a functional role for this sequence. Mutants of tdRSV with deletions in F1 have been constructed in vitro and deletion of most of F1 (only F1-D and part of F1-C remained) did not affect viral replication [123]. However, a deletion of the entire F1 extending into the polypurine tract abolished replication. These results support a functional role for either the polypurine tract or the last 15 bp of F1; the role of the bulk of F1 is at present unclear.

Undoubtedly, the presence of F1 in so many viruses facilitated recombination between various avian retroviruses. Viruses which were passaged together may have freely exchanged F1s and neighboring regions. Preliminary evidence [Bizub and Skalka, personal communication] suggests that such recombination occurred between BH-RSV and its helper virus, RAV-2. Examination of the F1 sequences in Fig. 2.8 reveals the fact that the F1 sequences of AMV and PR-RSV (between *env* and *src*) are remarkably similar, suggesting they may have once been passaged with a common helper virus.

RAV-60s are recombinant ALVs which contain an *env* gene acquired (in whole or in part) by recombination with endogenous viral sequences [48,52]; the U_3 region, however, is derived from the exogenous virus [23]. BH-RSV does not contain any sequences which are homologous to *env* but can give rise to RAV-

60s which have the *src* gene replaced by subgroup E *env* [48]. Generation BH-RSV-derived RAV-60s by recombination between BH-RSV and an ev provirus would, therefore, most likely involve crossing-over at homologous sites in the F1 sequence of BH-RSV and ev genomes. Another cross-over point in a homologous region upstream from *env* (most likely in *pol*) would also be required.

Chapter 3

The pol defect of BH-RSV alpha

INTRODUCTION

The pol gene is probably the most poorly understood of the retroviral genes. Its mRNA transcript has yet to be isolated (see introductory chapter), the processing pathway of the gag-pol precursor has not been directly shown, and the relationship between processing and virion assembly remains unclear. A number of functions have been attributed to the pol gene product: several enzymatic activities [115,142], roles in selection and packaging of cellular tRNAs [102,104,113], and a suspected role in integration of viral DNA. Unanswered questions still remain regarding the mechanism of reverse transcription of retroviral RNA; the exact role of the p32 endonuclease in viral replication as well as mechanisms for the other pol gene functions are as yet unknown.

Historically, mutants have been used extensively to study retroviral gene function. Many pol mutants have been isolated and have been at least partially characterized [for a review, see 84]. Since molecular cloning and rapid DNA sequencing techniques enable investigators to determine the nature of the defects more precisely, re-examination of pol mutants should result in new insights in understanding the function of the pol gene product.

In this study, I present characterization of the pol defect in BH-RSV alpha, a mutant with a stable pol$^-$ phenotype.

The results indicate that the defect appears to affect processing and/or packaging of the pol gene product. This mutant should therefore prove useful in further studies of these two pathways.

Transfection of viral DNA proved to be an invaluable assay for mapping the lesion. Since the original observation that viral DNA is transforming [55], the transfection assay has been used extensively to study retrovirus genetics and transformation. In this study, the requirement of viral replication for transformation of transfected CEF [22] was exploited to develop a bioassay for pol gene function. In vitro recombination techniques similar to those described in recent reports [98,116], were used to succesfully localize the pol defect to a discrete fragment within the pol gene.

RESULTS

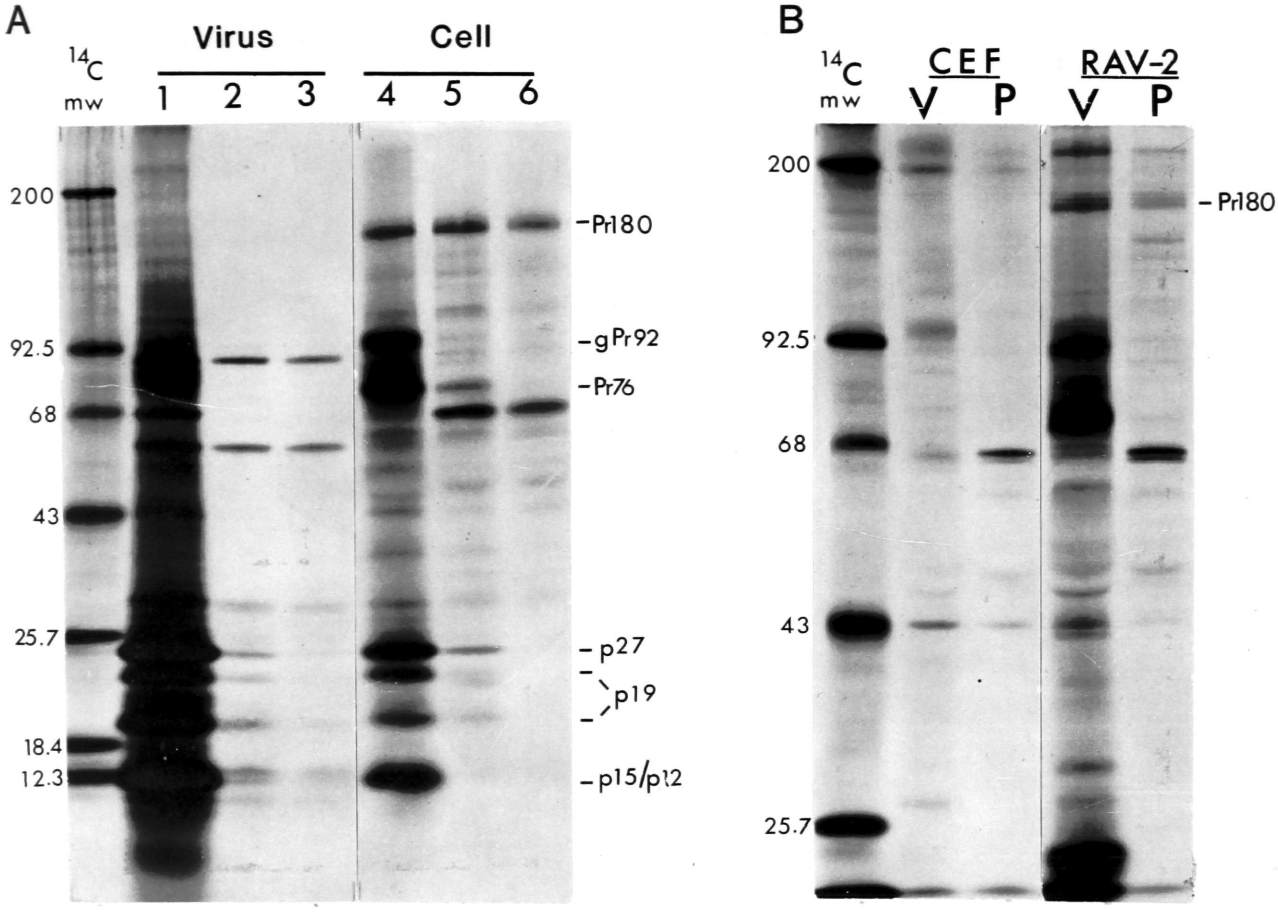
BH-RSV alpha-Infected Cells: Protein Studies

Antiserum controls. The BH-RSV alpha pol-related proteins synthesized either in the infected cells or packaged into virion particles were examined by immunoprecipitation followed by polyacrylamide gel electrophoresis. Antisera directed against whole virions or specific for reverse transcriptase were used; the characteristics of these antisera were determined by immunoprecipitation of proteins from RAV-2 virions and RAV-2-infected cultures. As shown in Fig. 3.1, panel A (lanes 2-3), reverse transcriptase antiserum (anti-pol serum) precipitated the reverse transcriptase subunits, $p95^{pol}$ and $p63^{pol}$, from virion particles. The serum also precipitated some viral structural proteins but most of this activity was removed by absorption of the serum with disrupted virus (compare lanes 2 and 3).

The anti-pol serum precipitated the gag-pol precursor Pr180 from infected cells (lanes 5 and 6); $p95^{pol}$ and $p63^{pol}$ were not detected in infected cells. The 67K protein which was precipitated with the absorbed anti-pol serum was a cellular contaminant as demonstrated in panel B.

Antiserum against whole virions (anti-virion serum) precipitated the gag-pol precursor Pr180 from infected cells (lane 4); the other viral proteins, $gPr92^{env}$, $Pr76^{gag}$, $p27^{gag}$, $p19^{gag}$, $p15/p12^{gag}$ were precipitated with this antiserum as well.

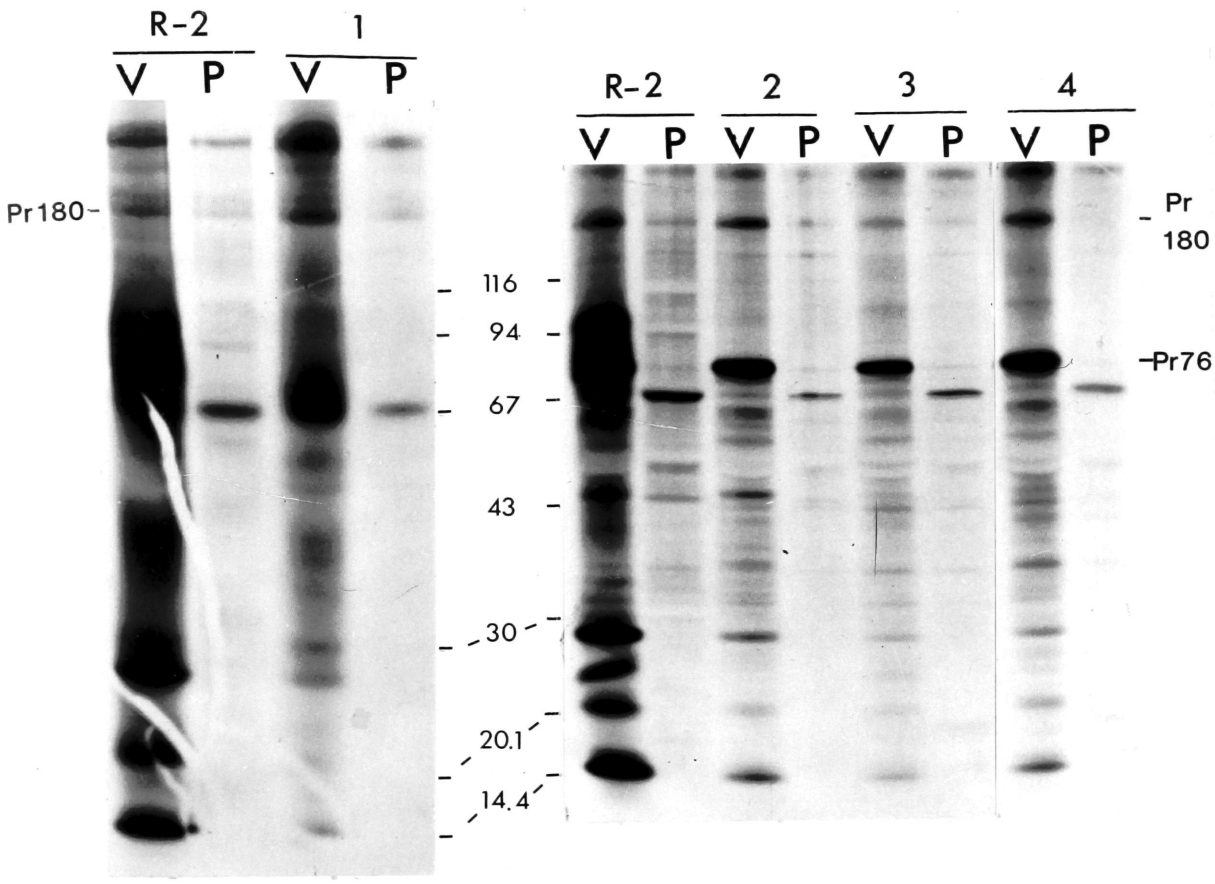
Figure 3.1. Characteristics of anti-pol and anti-virion sera. (A) Lanes 1 through 3 contain equal amounts of ^{35}S-methionine labeled RAV-2 virions which were purified as described in Materials and Methods. Lanes 4 through 6 contain immunoprecipitates of lysates of ^{35}S-methionine labeled RAV-2 infected cells. Lane 1, no immunoprecipitation; lanes 2 and 5, immunoprecipitation with anti-pol serum; lanes 3 and 6, immunoprecipitation with absorbed anti-pol serum; lane 4, immunoprecipitation with anti-virion serum. (B) Uninfected (CEF) or RAV-2-infected (RAV-2) cells were labeled with ^{35}S-methionine and cell lysates were prepared. Immunoprecipitation was performed with anti-virion serum (V) or anti-pol serum (P). Bands correpsonding to viral proteins are indentified on the right. Electrophoresis was performed on 7.5% SDS-polyacrylamide gels.


A
Virus
Cell
^{14}C
mw
1 2 3 4 5 6
200
92.5
68
43
25.7
18.4
12.3
-Pr180
-gPr92
-Pr76
-p27
-p19
-p15/p12
B
^{14}C
mw
CEF
RAV-2
V P V P
200
92.5
68
43
25.7
-Pr180

Viral proteins in BH-RSV alpha-infected cells. "Non-Producer" (NP) clones of CEF infected with BH-RSV alpha, free of helper virus, were isolated and the viral proteins were examined. Figure 3.2 shows the results of immunoprecipitation of viral proteins from extracts of BH-RSV alpha-infected cells using the anti-virion or anti-pol antiserum. Immunoprecipitates from RAV-2-infected cells run in parallel lanes are shown as controls. In all four NP clones, a band which co-migrates with the RAV-2 Pr180 was precipitated by both anti-virion and anti-pol serum. These results clearly indicate that BH-RSV alpha, although defective in polymerase, can direct the synthesis of a normal-sized gag-pol polyprotein precursor.

Immunoprecipitation with anti-virion serum (lanes marked "V") demonstrated the presence of the gag protein products in BH-RSV alpha-infected cells. As expected from the defectiveness of BH-RSV alpha in env, the env protein gPr92 was not detectable in the cell extracts from all the NP clones.

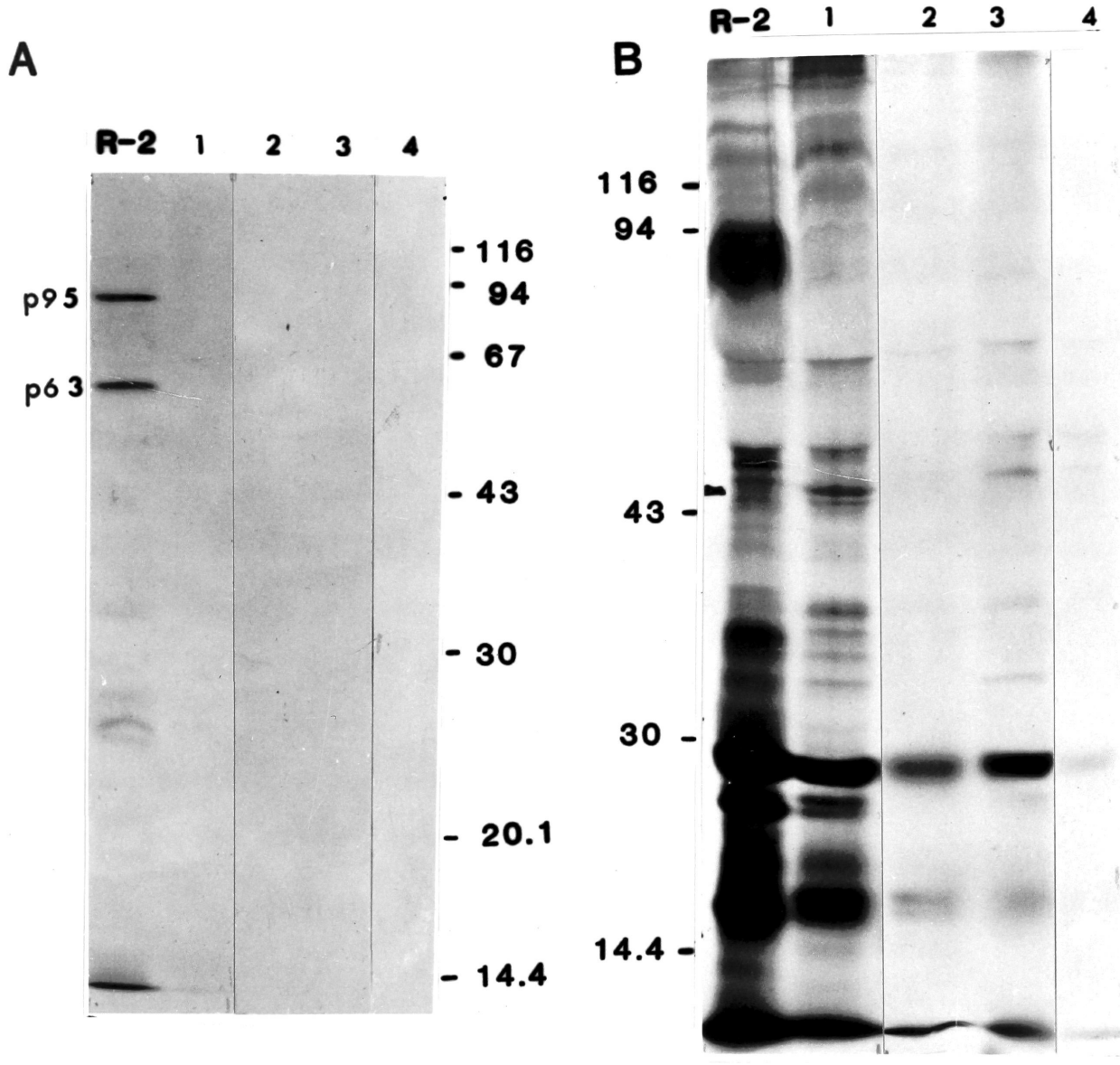
Figure 3.2. BH-RSV alpha proteins in infected cells. Cell lysates were prepared from cultures which had been labled with ^{35}S-methionine. R-2, RAV-2 infected mass culture; 1 through 4, independent BH-RSV alpha NP clones. Immunoprecipitation was performed with either anti-virion serum (V) or absorbed anti-pol serum (P). The positions of molecular weight markers run in parallel lanes of the gels are shown in the center. The bands corresponding to Pr76 and Pr180 are identified. Electrophoresis was performed on 5-15% SDS-polyacrylamide gradient gels.


R-2
1
V
P
V
P
Pr180
116
94
67
43
30
20.1
14.4
R-2
2
3
4
V
P
V
P
V
P
V
P
Pr
180
Pr76

Proteins in BH-RSV alpha virions As shown in Fig. 3.1, the mature reverse transcriptase subunits were not detected in immunoprecipitates of RAV-2-infected cells; however p95 and p63 were readily detectable in immunoprecipitates of purified RAV-2 virion particles. Figure 3.3 shows the results of immunoprecipitation of BH-RSV alpha virion particles. No polymerase-related proteins were detected in the virions released from at least 3 NP clones (panel A). In each of these 3 clones there was sufficient production of viral particles, as evidenced by the precipitation of gag proteins upon reprecipitatation with anti-virion serum (panel B), to rule out lack of virus production as the cause of the negative result. This confirms earlier results [44,101] in which a radioimmunoassay failed to detect any pol related products in BH-RSV alpha virions.

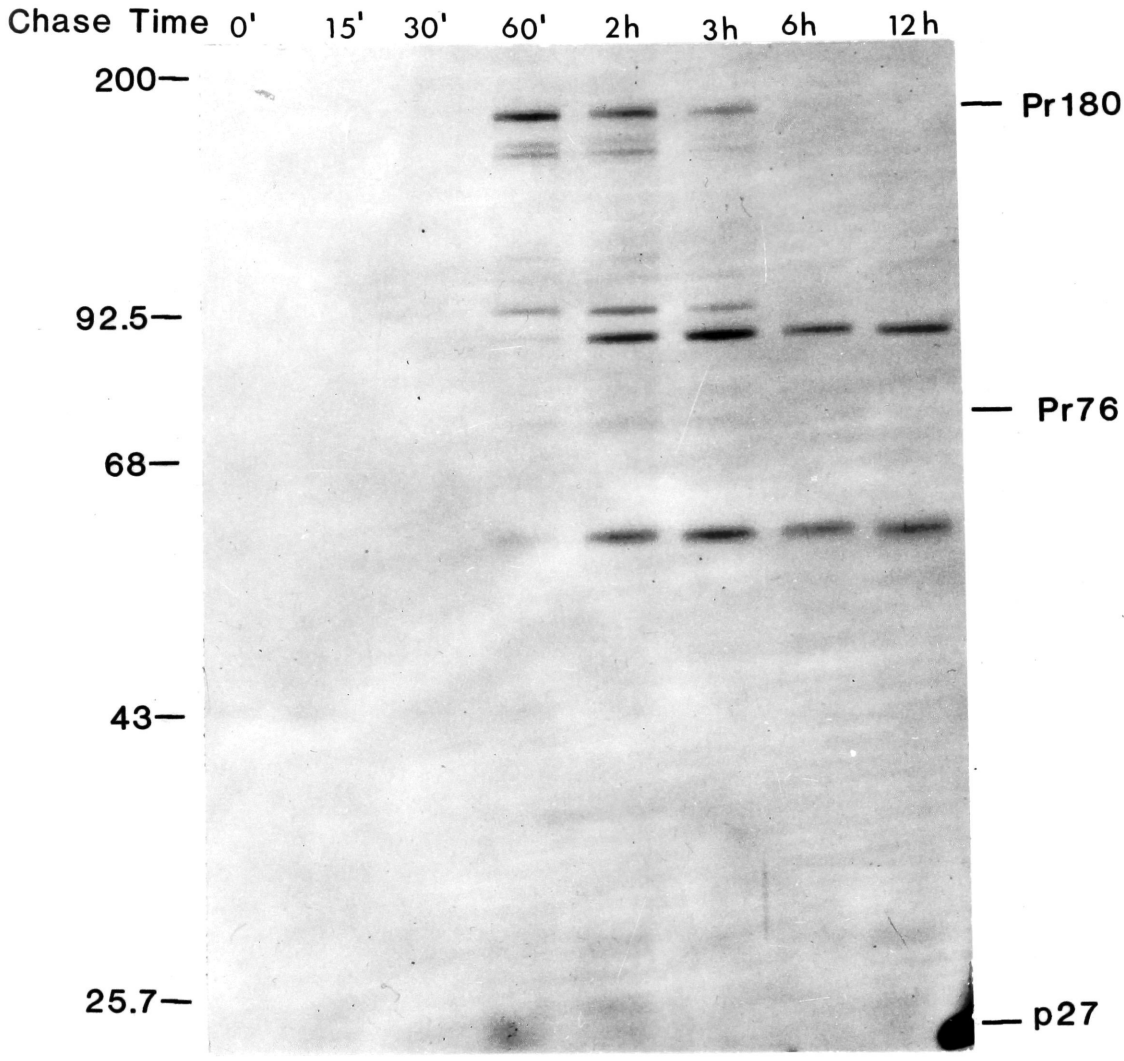
In conclusion, the pol-negative mutant, BH-RSV alpha, directs the synthesis of a full sized Pr180 gag-pol precursor molecule. However, this molecule is apparently not cleaved into the mature products p95 and p63 since BH-RSV alpha virion particles do not contain any polymerase-related proteins.

Figure 3.3 BH-RSV alpha proteins in purified virions. Purified ^{35}S-methionine labeled virion particles were immunoprecipitated with absorbed anti-pol serum (panel A). The material which did not precipitate was reprecipitated with anti-virion serum (panel B). The lane headings are the same as in Fig. 3.2. The positions of molecular weight markers run in parallel lanes of the gels are shown in the center. The bands corresponding to reverse transcriptase are identified. Electrophoresis was performed on 10% (panel A) or 5-15% (panel B) SDS-polyacrylamide gels.


A
R-2
1
2
3
4
p95
p63
116
94
67
43
30
20.1
14.4
B
R-2
1
2
3
4
116
94
43
30
14.4

Processing of Pr180. The processing pathway in the production of p95 and p63 from Pr180 is not well understood. Cultures of RAV-2-infected cells were used to determine whether processing of Pr180 occurs within virion particles. Cultures were labeled with ^{35}S-methionine for a short period, followed by a "chase" with unlabeled medium, according to the protocol described by Oppermann [100]. Culture fluid was harvested and ^{35}S-labeled virion particles were purified, immunoprecipitated with anti-pol serum, and examined by SDS polyacryalmide gel electrophoresis. The results (Figure 3.4) show that at early time points, (30-60 min. of chase), Pr180$^{gag-pol}$ was the major pol-related protein in virion particles. Within the second hour of the chase, the precursor form, Pr180, was processed into the mature form, p95 and p63. After 3 hours of chase, p95 and p63 were the major pol-related products in the virions. These data support the hypothesis that processing of Pr180 occurs exclusively in the virion particles; a similar hypothesis has been proposed for the processing of the gag-pol precursor of murine retroviruses [149].

Figure 3.4. Appearance of pol-related polypeptides in virions following pulse-labeling of RAV-2 infected cells. A 65 mm dish of RAV-2 infected CEF which had been starved of methionine for one hour was labeled with 1mCi of ^{35}S-methionine for 30 min. The medium was withdrawn, cells were washed, and fresh medium containing an excess of unlabeled methionine was added. The medium was withdrawn and replaced with fresh medium at the indicated time points. Virus was purified from each portion of medium and immunoprecipitated with anti-pol serum. (0'), the virus collected after the labeling period; (15'), the virus collected between 0-15 min after removal of the label; (30'), 30-60 min.; (2h), 1-2 hr; (3h), 2-3 hr; (6h), 3-6 hr; (12h) 6-12 hr. Electrophoresis was performed on a 7.5% SDS-polyacrylamide gel.


Chase Time 0' 15' 30' 60' 2h 3h 6h 12h
200—
92.5—
68—
43—
25.7—
Pr180
Pr76
p27

However when purified virion particles from culture supernatants were examined from cultures which were continuously labeled for various time periods (1 to 6 hr.), Pr180 was not detectable in the virion particles (data not shown). Although the labeling conditions in the two experiments differed and this may account for the discrepancy (1 mCi was used in the experiment in which Pr180 was detected in the virions and 300 uCi was used in the experiment in which it was not detected), further work must be done to elucidate the mechanism of Pr180 processing.

Characterization of BH-RSV alpha on the DNA Level

Since the BH-RSV alpha viral genome directs the synthesis of an apparently normal Pr180 precursor and since no obvious defects in the structure of the BH-RSV alpha pol gene were detected by restriction enzyme mapping, I sought to determine whether the defect actually mapped in the pol gene. The biological activity of the BH-RSV alpha pol gene was tested in a transfection assay. As a control, the activity of the BH-RSV beta pol gene was examined in a similar manner.

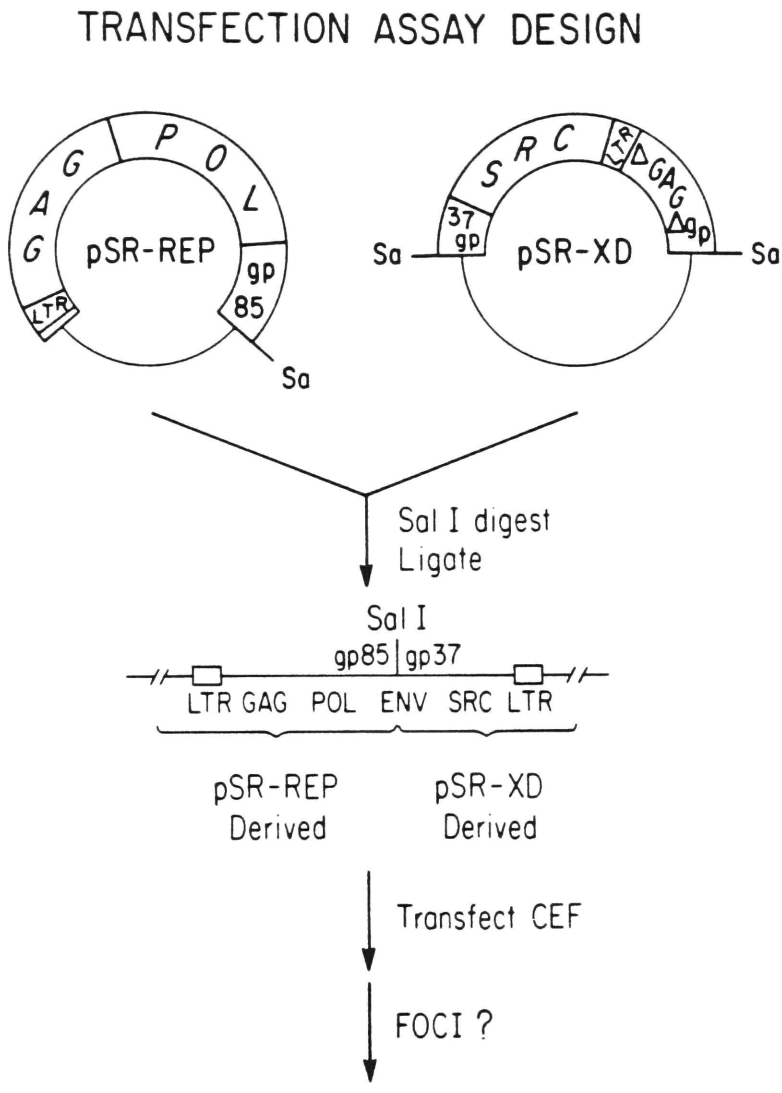


Figure 3.5. Transfection assay design. pSR-REP and pSR-XD are derived from pSRA, a pBR322 molecular clone containing the entire genome of SR-RSV [24]. After SalI digestion and ligation of the fragments of the two clones, the ligated mixture is transfected onto CEF (see Materials and Methods). One of the ligation products will be a structure equivalent to an SR-RSV provirus. Sa, position of SalI sites on the plasmids.

The transfection assay of CEF as designed by Cross and Hanafusa [24] was used (Figure 3.5). Two SR-RSV molecular clones, pSR-REP and pSR-XD, are ligated prior to transfection to generate an intact SR-RSV proviral genome. pSR-REP contains the SR-RSV 5' LTR, gag and pol genes, and the gp85-portion of the env gene; pSR-XD contains the SR-RSV gp37-portion of env, the src gene, and the 3' LTR. Neither plasmid alone can cause transformation of CEF since viral replication is required for stable transformation of transfected CEF [22].

Construction of SR-RSV/BH-RSV chimeras. Because of the requirement of viral replication for stable transformation, it was possible to use morphological transformation as a marker for assaying the biological activity of pol. Construction of in vitro recombinants between the pSR-REP clone and pBH-alpha or beta clones resulted in generation of viral DNA which contained all of its genes and regulatory sequences from SR-RSV except for a pol gene which was derived from BH-RSV alpha or beta. The strategy for construction of these recombinants is shown in Figure 3.6. The EcoRI-KpnI fragment contained the gag-pol junction and more than 90% of the pol gene (the portion of the pol gene between the KpnI site and the termination codon had already been partially sequenced and was found to be essentially identical in the alpha and beta strains (Fig. 2.5)).

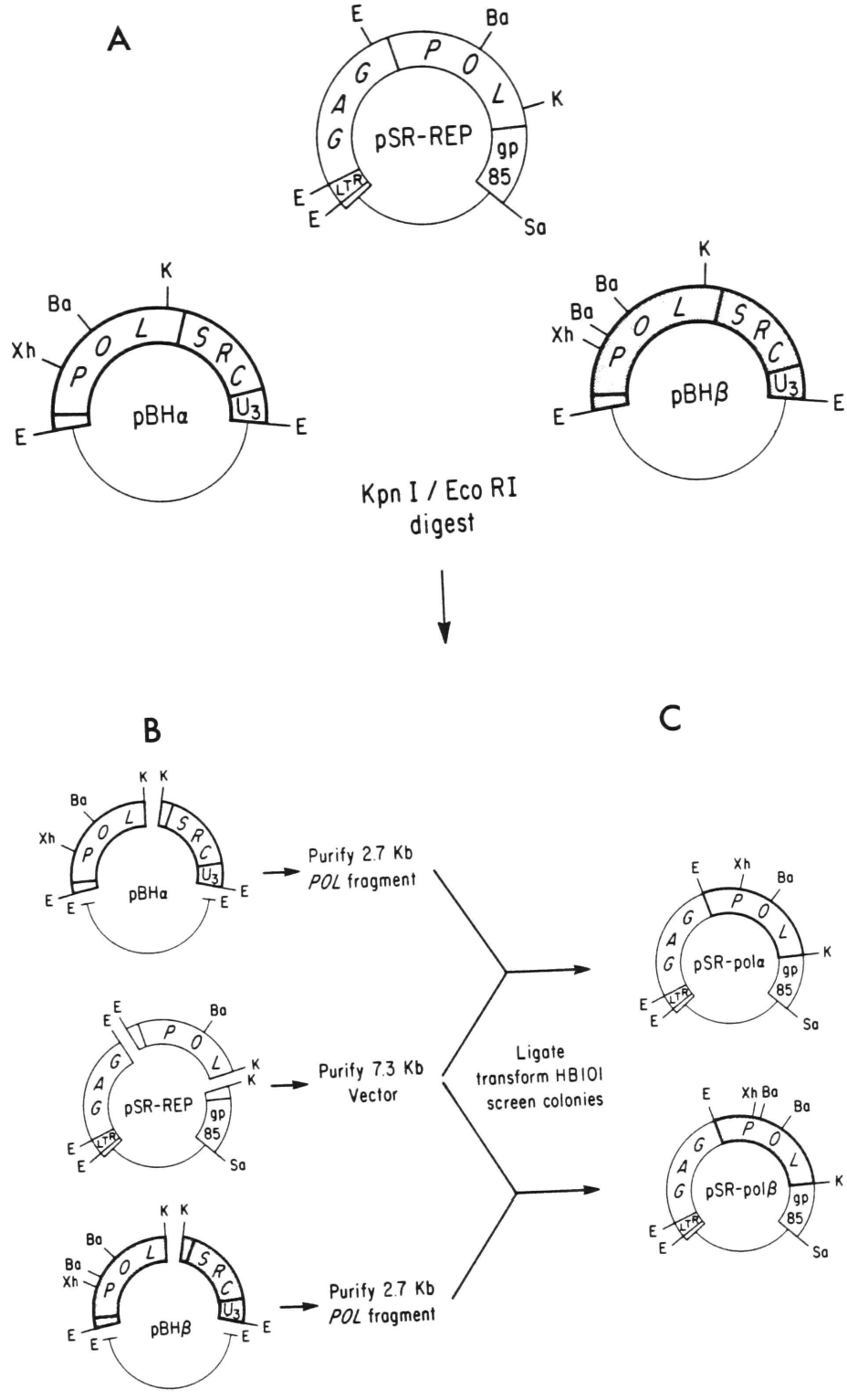


Figure 3.6. Construction of SR-RSV/BH-RSV chimeric clones. (A) Structure of molecular clones which were used to derive the chimeric constructions [24, and Chapter 2, this thesis]. (B) Products of EcoRI and KpnI digestions described in the text. (C) Structures of resulting recombinants. The positions of relevant restriction enzyme cleavage sites are shown. For simplicity, in (C) and in Figs. 3.7 and 3.8, the EcoRI and KpnI sites are shown at the 5' and 3' borders of pol. E, EcoRI; K, KpnI; Xh, XhoI; Ba, BamHI.

The EcoRI-KpnI pol-containing restriction fragment was excised from pBH-alpha or pBH-beta DNA by complete digestion with KpnI and EcoRI. The excised fragment was ligated to pSR-REP DNA which had been linearized by complete digestion with KpnI and then had the pol-containing EcoRI-KpnI fragment removed by partial digestion with EcoRI. DNA of the in vitro-constructed pol recombinants was amplified by transformation of E. Coli; colonies were then screened for plasmids of the desired construction. Since both BH-RSV strains contained an XhoI site in pol which was not present in SR-RSV, and since BH-RSV beta contained an extra BamHI site in pol which was not present in BH-RSV alpha or SR-RSV (Fig. 2.3), these two enzymes were used to determine the origin of the EcoRI-KpnI pol fragment in the recombinants. The resulting recombinants, pSR-pol-alpha and pSR-pol-beta, had the constructions shown in Fig. 3.6C.

Mapping the defect to the pol coding sequences. DNA from pSR-REP, or the recombinants pSR-pol-alpha or pSR-pol-beta, was ligated to pSR-XD DNA and transfected onto chicken embryo fibroblasts as shown schematically in Figure 3.5. Replacement of the SR-RSV pol gene with the BH-RSV alpha pol gene abolished focus-forming ability, while replacement of the SR-RSV pol gene with that of BH-RSV beta did not affect focus-forming ability (Table I). Therefore, the defect which is responsible for the lack of acitivity in the transfection assay and presumably, for the lack of reverse transcriptase enzyme in BH-RSV alpha virion particles, maps within the EcoRI-KpnI fragment of BH-RSV alpha.

TRANSFECTION ASSAY

Viral DNA		RESULT	
5' portion	3' portion	FOCI	Remarks
pSR-REP	pSR-XD	+	Total transformation after 8 days
pSR-polα	pSR-XD	-	No foci after 8 days
pSR-polβ	pSR-XD	+	Total transformation after 5 days
-----	pSR-XD	-	No foci after 8 days

Table I. Transfection of CEF with viral DNAs. In each experiment, 4 ug of each DNA species was used for transfection of four 65 mm dishes of CEF, according to the protocol outlined in Fig. 3.5. Cultures were transfered and overlaid with agar as described in the Materials and Methods and monitored for the appearance of foci.

Construction of recombinants within the pol gene. The XbaI and BglII cleavage sites within the EcoRI-KpnI fragment, divide the 2.7 kb fragment into three fragments of 1.07, 0.86, and 0.76 kb. Figure 3.7 shows the strategy used for constructing recombinants which would enable the pol defect to be mapped to one of these three fragments.

pSR-pol-alpha and pSR-pol-beta were used as parentals. The DNAs were linearized by complete digestion with the single-cut enzyme SstI and partial digest conditions were used to generate SstI-XbaI or SstI-BglII fragments cut in pol (Fig. 3.7B). The SstI-XbaI and SstI-BglII "vector" (larger) and "insert" (smaller) fragments from pSR-pol-alpha and pSR-pol-beta were each cut from an agarose gel and purified separately. To remove undigested or nicked circular plasmid (i.e., parental) DNA which may comigrate with the vector fragments, purified vector fragments were further digested with HpaI. HpaI cleaves parental DNA at a single site which maps within the fragment which is not present in the two vectors (Fig. 2.3).

For both the SstI-XbaI and SstI-BglII digestion products, the pSR-pol-alpha vector was ligated to the pSR-pol-beta insert and the pSR-pol-beta vector was ligated to the pSR-pol-alpha insert, resulting in the construction of the four pol recombinants illustrated in Figure 3.7C. BamHI, which differentiates the BH-RSV alpha- and beta-derived inserts, was used to verify the constructions.

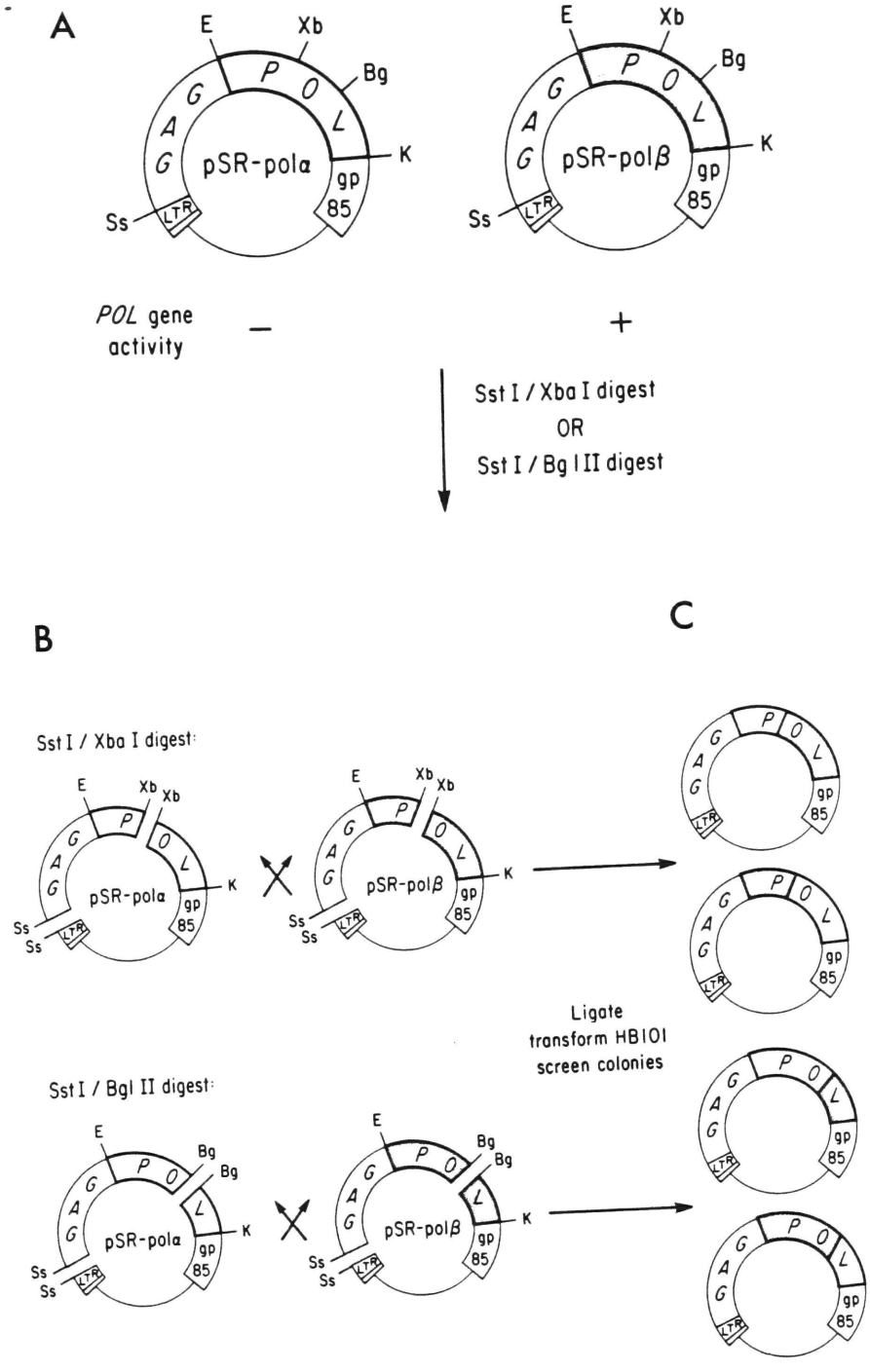


Figure 3.7. Construction of recombinants within pol. (A) Structure of molecular clones which were used to derive the recombinants. Activity of the pol gene was based on data in Table I. (B) Products of SstI-XbaI or SstI-BglII double digestions described in the text. (C) Structures of resulting recombinants. In (A) and (B) the positions of relevant restriction enzyme sites are shown. Ss, SstI; Xb, XbaI; Bg, BglII; E, EcoRI; K, KpnI.

Localization of the defect to a defined region of pol gene. The four recombinants shown in Fig. 3.7 were used in the DNA transfection assay to unambiguously map the pol defect to either the 1.07 kb EcoRI-XbaI ("P") fragment, the 0.86 kb XbaI-BglII ("O") fragment, or the 0.76 kb BglII-KpnI ("L") fragment. The gag-pol junction maps in the "P" region; the site at which $p95^{pol}$ is cleaved into $p63^{pol}$ and p32 is thought to map in the "O" region. As shown in Figure 3.8, given that the alpha-derived pol fragment is negative and the beta-derived fragment is positive in the transfection assay, the four recombinants which were constructed would yield a unique array of results depending on whether the mutation mapped in "P", "O", or "L". (Other unique patterns of results which would be expected if more than one mutation existed are not shown in the figure.)

When the four recombinants were tested in the transfection assay, successful transformation of CEF correlated with a beta-derived "O" region only. Conversely, inability to produce foci on CEF, correlated with an alpha-derived "O" region irrespective of the origin of the "P" or "L" fragments (Fig. 3.8).

Thus, the defect in BH-RSV alpha which results in a Pr180 protein which does not seem to be processed is due to a mutation in the coding sequence of its pol gene. The mutation maps to an 859 bp XbaI-BglII fragment in the central portion of the gene. Sequencing of this region is currently in progress.

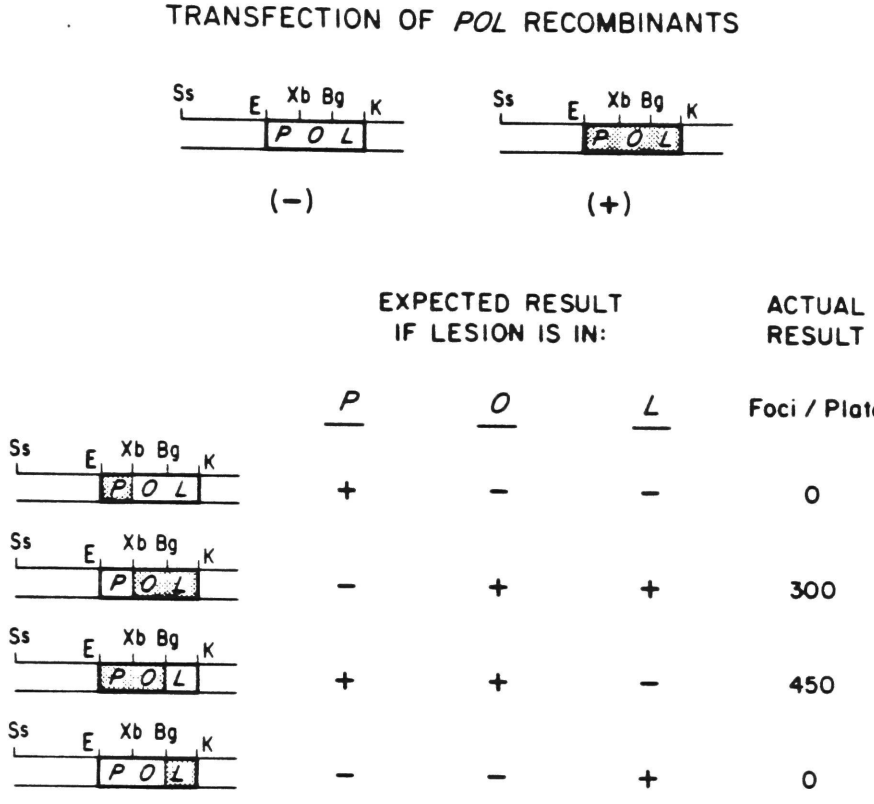


Figure 3.8. Transfection of pol recombinants. The structure and biological activity of the pol gene of pBH-alpha (upper left) and pBH-beta (upper right) are shown for reference. The structure of the pol genes of the four recombinants derived in Fig. 3.7 and the expected results in a transfection assay, assuming that the pol lesion mapped to one of the three indicated sections of the pol gene, are shown in the table. The actual results were the average of the results of transfection of 3 independent molecular clones having the structure shown, except the third row which was the average of the results of two clones.

Difference between the pol gene of SR-RSV and Bryan RSV beta. Both SR-RSV and BH-RSV beta have wild-type pol genes which would be expected to be equally active in a transfection assay. It appeared that pSR-pol-beta, the construct in which the pol gene of SR-RSV was replaced with that of BH-RSV beta, was more active in the transfection assay than the parental pSR-REP (Table I). pSR-pol-beta consistently (more than 5 independent experiments) caused rapid and complete transformation of the cultures in 5 days, while under the same conditions, cells transfected with pSR-REP did not begin to show discernable foci until day 6, and the plates did not become completely transformed until (at least) day 7.

This observation was confirmed by the measurement of the kinetics of production of infectious viral particles by pSR-REP- and pSR-pol-beta-transfected cultures. Culture supernatants collected daily, beginning 4 days post-transfection, were used to determine the titer of infectious viral particles. As can be seen in Figure 3.9, the pSR-pol-beta-transfected cultures consistently contained 10-100 fold more infectious particles than the pSR-REP cultures; even the maximum titer attained by the two cultures differed by at least a factor of 10.

Replacement of the 2.7 kb EcoRI-KpnI pol fragment of SR-RSV with that of BH-RSV beta resulted in an enhancement of viral replication. Many functions have been attributed to the retrovirus pol gene, and we do not know which function in BH-RSV contributes to such a dramatic stimulation of infectous virus.

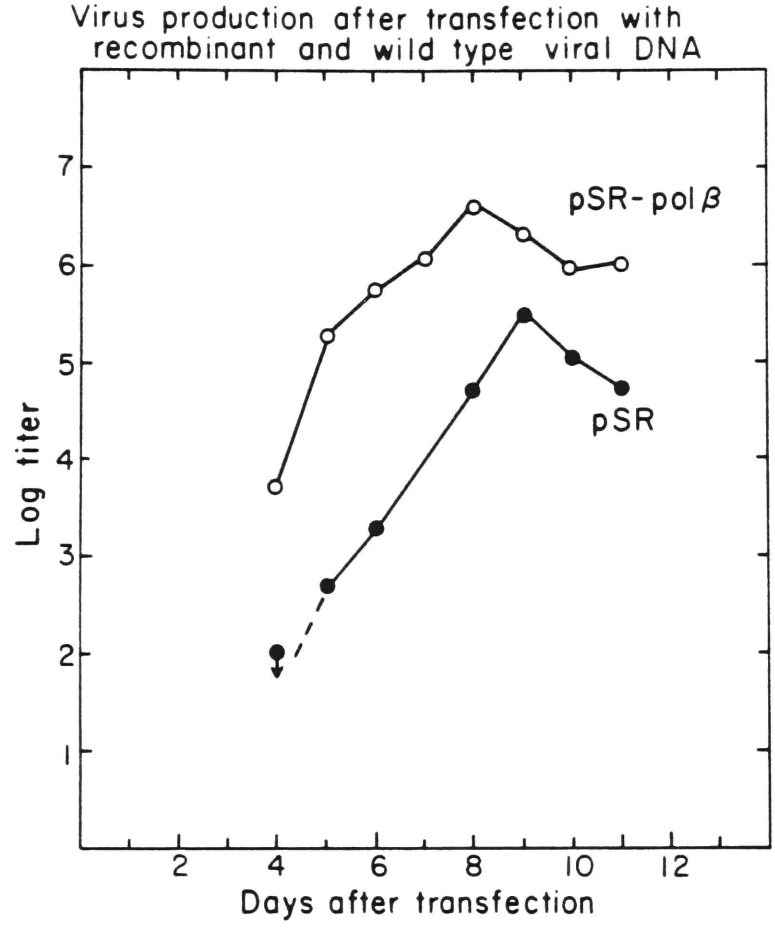


Figure 3.9. Titer of infectious virus in culture fluid of cells transfected with viral DNA. SalI-cut pSR-REP (filled circles) or pSR-pol-beta (open circles) DNA was ligated to pSR-XD DNA and ligated DNA was transfected onto CEF. Beginning four days after transfection, culture fluid was collected daily and clarified fluid was used to determine the number of focus forming units (ffu)/ml. For pSR-REP-transfected cultures, the titer on day 4 was below detection limits of the assay (100 ffu/ml).

DISCUSSION

Polymerase Defect in BH-RSV alpha

Restriction enzyme mapping studies using a large number of enzymes found the structure of the BH-RSV alpha *pol* gene to be almost indistinguishable from that of wild type *pol* genes (Figs. 2.2 and 2.3). Protein studies indicated that although a full length *gag-pol* precursor was synthesized, neither the mature processed *pol* products nor any other polymerase related proteins were detected in BH-RSV alpha virions (Figs. 3.2 and 3.3). In this respect, the *pol* defect in BH-RSV alpha seems to be quite different than that of other non-conditional *pol* mutants which have been examined biochemically.

The *pol*$^-$ phenotypes in mutants Q-PH9 and SE52d, and in the provirus ev3 are due to deletions in the respective *pol* genes; all three synthesize a truncated form of Pr180 (P140, P125, and P120 respectively) in infected cells and, with the exception of ev3 which does not produce viral particles, they do not produce reverse transcriptase protein in their viral particles [33,34,85,90].

There does not appear to be a deletion or major sequence rearrangement affecting the *pol* sequences of the ev-1 provirus [60,125], yet like BH-RSV alpha, ev-1 (under conditions where it is expressed) virion particles do not contain reverse transcriptase-related proteins [21]. However, immunoprecipitation of cell lysates showed that ev-1-expressing cells do not synthesize *any* intracellular *gag-pol* product [21]. The cause of the ev-1 *pol* defect has not been determined.

The AMV genome directs the synthesis of a full size $Pr180^{gag-pol}$ protein, yet virion particles contain no reverse transcriptase-related proteins [32]. Although this seems to be phenotypically identical to the BH-RSV alpha pol defect, the cause of the AMV defect is now understood. Recent DNA sequence data has demonstrated that the pol defect in AMV is due to insertion of the myb gene into the 3' end of pol (between the KpnI site and the termination codon) [78,110]. As a result, the last 36 amino acids of AMV pol are probably derived from c-myb [78]. However, DNA sequencing of BH-RSV alpha demonstrated that this portion of the pol gene was not significantly different than the wild type pol gene (Fig. 2.5).

Other models to explain the BH-RSV alpha pol defect must therefore be considered:

1) Defect in splicing pol mRNA: The avian gag and pol gene products are encoded on different reading frames of viral RNA [117] and a splicing event is presumably required to remove the gag translation terminator and shift the reading frame. Suppression of the gag terminator and continued translation in the gag reading frame has been shown to result in a read-through protein of 80 kD [147]. A shift to the incorrect reading frame would presumably result in an even smaller read-through protein [based on 117]. Therefore, the presence of a full size BH-RSV alpha gag-pol precursor protein, containing pol antigenic determinants, argues against a defect in the splicing event. However, a splicing defect may explain the nature of the ev-1 defect (see above) [21].

2) Lesion outside of pol: Consistent with the protein and restriction mapping data is the hypothesis that the defect in BH-RSV alpha affects its reverse transcriptase protein but does not map within pol. A possible candidate for such a defect would be the protease responsible for processing of Pr180. $p15^{gag}$ protease has been implicated in virus maturation [29,143,144]. In vitro, p15 cleaves Pr180 non-specifically [34,94], however, this does not rule out the possibility of a role for p15-mediated processing of Pr180 in vivo. In fact, p15 has been shown to cleave $p95^{pol}$ quite specifically into $p63^{pol}$ and a 32 kD protein [94]. Assuming that processing of Pr180 occurs prior to packaging, BH-RSV may be a processing mutant due to a defective protease (p15 or another, as yet unidentified, viral activity involved in processing) or due to an alteration of a cleavage site(s).

3) Defect in packaging: Preliminary evidence presented in Fig. 3.3 indicates that unprocessed Pr180 may be the moeity which is packaged into the virions. In that case, a processing defect cannot account for the lack of detectable reverse transcriptase-related proteins in virion particles. Even if BH-RSV alpha Pr180 was not cleaved or cleaved only partially, I would have expected to detect the accumulation of uncleaved Pr180 or one of its processing intermediates in virion particles.

If packaging of Pr180 is indeed a prerequisite for processing, BH-RSV alpha may be a packaging mutant with a lesion in pol which renders the Pr180 protein unpackageable. Alterna-

tively, if an interaction between the pol proteins and the virion core proteins or the viral RNA is required for packaging of the enzyme, a defect in gag or in a non-coding region of viral RNA could produce the observed phenotype. Although there is no evidence that such interactions, are required, it is known that the pol gene product binds $tRNA^{trp}$ [102] and seems to be responsible for selecting a specific cellular tRNA pool for packaging [104,113].

4) Artifact of molecular cloning: The protein analysis of BH-RSV alpha was performed on many independent clones of NP cells (Figs 3.2, 3.3, and data not shown), whereas the restriction mapping was performed on one molecular clone. The possibility that the pBH-alpha clone was not representative of the BH-RSV alpha genome also had to be considered.

The transfection assay resolved many of these points. The biological activity of molecularly cloned DNA fragments used in this study was confirmed. The reverse transcriptase defect in BH-RSV alpha was localized to a specific fragment within the BH-RSV alpha pol gene, effectively eliminating the possibility that the lesion mapped outside of pol or in the gag-pol junction. Until the DNA sequence of the defective region is available, the exact nature of the pol defect is still a matter for speculation.

The cleavage site used in processing p95 into p63 and p32 is thought to map within the defective region [D. Grandgenett, personal communication]. While such a defect would certainly affect processing of reverse transcriptase, whether it could

also affect packaging of pol products is not clear. However, if the defect causes a major alteration in the protein sequence, a conformational change affecting Pr180 (or p95) may prevent proper packaging.

The pol defect in AMV may also be related to packaging. It does not seem likely that the proposed carboxy terminal substitution in the AMV Pr180 protein affects any of the cleavage sites used in processing of this protein. Since pol (or pol-related) proteins are not detected in viral particles, the resulting alteration in protein structure may prevent proper packaging. Further studies on the fate of the BH-RSV and AMV Pr180 proteins should be useful in establishing the relationship between processing and packaging.

Proposed mechanisms for the generation of the BH-RSV alpha defect must account for both the frequency of its occurance (5-10%) and its low rate of spontaneous reversion [46]. A point mutation is unlikely to be stable. An in-frame deletion would be consistent with the evidence presented, provided the deletion was small enough to be undetectable by the restriction enzyme mapping analysis. Another possibility is that the BH-RSV alpha pol defect is generated by recombination. Almost all the ev proviruses contain some pol sequences [53]. Since earlier studies found the frequency of generation of the BH-RSV alpha defect to be independent of the gs or chf phenotype of the embryo [112], a recombination model would have to assume that there is a defective portion of pol which is conserved among most of the ev's, or would have to assume recombination

involved the commonly found provirus ev-1. It is possible that ev-1 contains an internal defect in pol, in addition to the proposed splicing defect (see above).

Processing of Pr180

Whereas mature reverse transcriptase protein and enzymatic activity are readily detectable in virion particles, many workers have reported the inability to detect the reverse transcriptase subunits or reverse transcriptase enzymatic activity in retrovirus-infected cells [34,51,100,149]. It is therefore believed that cleavage of Pr180 and formation of mature pol products occurs very close to the time of release of virion particles from the infected cells. However, whether cleavage occurs before, after, or during the budding process has not been resolved. Although there is general agreement regarding the absence of detectable amounts of p95 and p63 in infected cells, the presence of Pr180 in mature virion particles is the subject of conflicting reports.

In the report in which the avian $Pr180^{gag-pol}$ was initially described, Oppermann et al [100] note the presence of high molecular weight bands in polyacrylamide gels containing proteins from purified virion particles and suggest that one of these bands represents Pr180. This high molecular weight protein comigrates (on their gel system, i.e., 11% polyacrylamide) with Pr180 found in infected cells and appears in pulse-chase experiments only in virion particles harvested at early time points after removal of the radioisotope. However immune precipitation was not used to firmly establish the identity of

this band as the pol precursor.

Moelling et al [94] report the presence of Pr180 in Coomassie blue-stained polyacrylamide gels of disrupted virion particles. Virion "Pr180" was identified by comigration with cellular Pr180; immune precipitation was not used.

Eisenman et al [34] report the absence of Pr180 in radioactively labeled virion particles which were immunoprecipitated with anti-reverse transcriptase serum. Virions were purified from cultures which had been labeled continuously for 1-6 hr. These workers also report (although the data is not shown) that Pr180 could not be immunoprecipitated from virions purified from cultures which they had labeled according to the pulse-chase protocol described by Oppermann et al.

In an attempt to clarify this conflict, I repeated the experiments of Oppermann et al and Eisenman et al. In order to verify the identity of high molecular weight bands which may appear in the virions, immunoprecipitation with anti-pol serum was used in repeating Oppermann's experiment. In our experiments in which parallel cultures of infected cells were used, the contradictory reports of both sets of workers were confirmed; Pr180 was clearly identified in virions in the pulse-chase experiment and was undetectable in the continuous labeling experiment (Fig. 3.4, and data not shown). These results must therefore be regarded as preliminary and resolution of the question of packaging of Pr180 will require further investigation.

Temperature sensitive packaging mutants Mo-MuLV ts3 [150]

and Ra-MuLV ts24 [131] were used to study the maturation of murine retroviruses. These studies [149] suggest that the sequence of events in late stages of assembly is: encapsidation of uncleaved $Pr180^{gag-pol}$ followed by cleavage into reverse transcriptase in newly formed virions. Processing of Pr180 appears to occur rapidly, within 40 min of release of the virion particle. These workers suggest that activation of reverse transcriptase in virion particles may reflect a general mechanism whereby premature reverse transcription of viral RNA is prevented from occurring within the cell. For this reason, activation of reverse transcriptase (by cleavage) within virion particles would be advantageous to avian retroviruses as well.

Enhanced viral production by the pol gene of BH-RSV beta

It is not clear which function of the BH-RSV beta pol gene product accounts for the increased production of infectious virus by cells which were transfected by pSR-pol-beta as compared to pSR-REP (Fig. 3.9). Possibly, the rate of one of the enzymatic activities of the reverse transcriptase protein is increased. Alternatively, assuming that packaging of reverse transcriptase (either as Pr180 or as p95/p63) or selection of $tRNA^{trp}$ and/or binding of it to viral RNA are somewhat inefficient processes, the BH-RSV pol gene product may have a structural alteration which increases its efficiency. In such a model, the ratio of infectious to physical viral particles would be higher in viruses containing the BH-RSV pol gene.

Further work will have to be done to understand this puzzling phenomenon. The virus harvested from the supernatants of

pSR-REP and pSR-pol-beta transfected cultures should be examined to determine the kinetics of production of infectious particles early after infection. Also, to rule out the possibility that the pol gene of pSR-REP may have incurred a defect during molecular cloning, SR-RSV virus should be included for comparison in future experiments. It would also be interesting to examine the purified enzyme encoded by BH-RSV beta pol. If the efficiency of synthesis or fidelty of the transcript is substantially increased in comparison to other viral polymerases, this enzyme may be valuable for in vitro uses of reverse transcriptase (i.e., cDNA cloning).

REFERENCES

1. Abelson, H.T. and L.S. Rabstein. 1970. Lymphosarcoma: virus induced thymic-independent disease in mice. Cancer Res. 30:2213-2222.

2. Astrin, S. 1978. Endogenous viral genes of the white leghorn chicken: Common site of residence and sites associated with specific phenotypes of viral gene expression. Proc. Natl. Acad. Sci. USA 75:5941-5945.

3. Astrin, S., H.L. Robinson, L.B. Crittenden, E.G. Buss, J. Wyban, and W.S. Hayward. 1980. Ten genetic loci in the chicken that contain structural genes for endogenous viruses. Cold Spring Harbor Symp. Quant. Biol. 44:1105-1109.

4. Bacheler, L.T., and H. Fan. 1979. Multiple integration sites for Moloney murine leukemia virus in productively infected mouse fibroblasts. J. Virol. 30:657-667.

5. Becker, A. and M. Gold. 1975. Isolation of the bacteriophage lambda A-gene protein. Proc. Natl. Acad. Sci. USA 72: 581-585.

6. Benton, W.D. and R.W. Davis. 1977. Screening lambda gt recombinant clones by hybridization to single plaques in situ. Science 196: 180-182.

7. Bergmann, D.G., and M.A. Baluda. 1980. DNA of avian myeloblastosis associated virus type 2 integrates at multiple sites in chicken genome. J. Virol. 35:9968-9971.

8. Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening reecombinant plasmid DNA. Nucl. Acids Res. 27:1513-1523.

9. Bolivar, F.R., L. Rodriguez, P.J. Greene, M.C. Betlach, H.L. Heynekeer, and H.W. Boyer. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95-113.

10. Boone, L.R. and A.M. Skalka. 1981. Viral DNA synthesized in vitro by avian retrovirus particles remeabilized with mellitin. I. Kinetics of synthesis and size of minus- and plus-strand transcripts. J. Virol. 37:109-118.

11. Botchan, M., W. Topp, and J. Sambrook. 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. Cell 9:269-287.

12. Boyer, H.W. and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in Escherichia coli. J. Mol. Biol. 41:459-472.

13. Brugge, J.S. and R.L. Erikson. 1977. Identification of

a transformation specific antigen induced by avian sarcoma virus. Nature (London) 269:346-348.

14. Bryan, W.R. 1959. Enhancement of virus yeild of Rous sarcoma virus. Acta Un. Int. Cancer 15:764-767.

15. Campbell, A. 1962. Episomes. Adv. Genet. 11:107-145.

16. Coffin, J.M., M. Champion, and F. Chabot. 1978. Nucleotide sequence relationships between the genomes of an endogenous and an exogenous avian tumor virus. J. Virol. 28:972-991.

17. Coffin, J.M., H.E. Varmus, J.M. Bishop, M. Essex, W.D. Hardy, G.S. Martin, N.E. Rosenberg, E.M. Scolnik, R.A. Weinberg, and P.K. Vogt. 1981. Proposal for naming host cell-derived inserts in retrovirus genomes. J. Virol. 40:953-957.

18. Cohen, J.C., P.R. Shank, V.L. Morris, R. Cardiff, and H.E. Varmus. 1979. Integration of the DNA of mouse mammary tumor virus in virus infected normal and neoplastic tissue of the mouse. Cell 16:333-345.

19. Collins, C.J., D. Boettinger, T.L. Green, M.B. Burgess, B.H. Devlin, and J.T. Parsons. 1980. Arrangement of integrated avian sarcoma virus DNA sequences within the cellular genomes of transformed and revertant mammalian cells. J. Virol. 33:760-768.

20. Collins, C.J., and J.T. Parsons. 1977. Integration of

avian sarcoma virus DNA sequences in transformed mammalian cells. Proc. Natl. Acad. Sci. USA 74:4301-4305.

21. Conklin, K.F., J.M. Coffin, H.L. Robinson, M. Groudine, and R. Eisenman. 1982. Role of methylation in induced and spontaneous expression of the avian endogenous virus ev-1: DNA structure and gene products. Mol. Cell. Biol. 2:638-652.

22. Cooper, G.M., and S. Okenquist. 1978. Mechanism of transfection of chicken embryo fibroblasts by Rous sarcoma virus DNA. J. Virol. 28:45-52.

23. Crittenden, L.B., W.S. Hayward, H. Hanafusa, and A.M. Fadly. 1980. Induction of neoplasms by subgroup E recombinants of exogenous and endogenous avian retroviruses (Rous associated virus type 60). J. Virol. 33:915-919.

24. Cross, F.R., and H. Hanafusa. 1983. Local mutagenesis of Rous sarcoma virus: the major sites of tyrosine and serine phosphorylation of p60-src are dispensable for transformation. Cell, submitted.

25. Davis, R.W., D. Botstein, and J.R. Roth, eds. 1980. Advanced Bacterial Genetics. Cold Spring Harbor Laboratory (New York).

26. DeLorbe, W.J., P.A. Luciw, H.W. Goodman, H.E. Varmus,

and J.M. Bishop. 1980. Molecular cloning and characterization of avian sarcoma virus circular DNA molecules. J. Virol. 36:50-61.

27. Dhar, R., W.L. McClements, L.W. Enquist, and G.F. Vande Woude. 1980. Nucleotide sequences of integrated Moloney sarcoma provirus long terminal repeats. and their host and viral junctions. Proc. Natl. Acad. Sci. USA 77:3937-3941.

28. Dina, D. and E.W. Benz. 1980. Structure of murine sarcoma virus DNA replicative intermediates synthesized in vitro. J. Virol. 33:377-389.

29. Dittmar, K.J., and K. Moelling. 1978. Biochemical properties of p15-associated protease in avian RNA tumor virus. J. Virol. 28:106-118.

30. Duesberg, P., S. Kawai, L-H Wang, P.K. Vogt, H.M. Murphy, and H. Hanafusa. 1975. RNA of replication defective strains of Rous sarcoma virus. Proc. Natl. Acad. Sci. USA 72:1569-1573.

31. Duesberg, P.H. and P.K. Vogt. 1973. RNA species obtained from clonal lines of avian sarcoma and from avian leukosis viruses. Virology 54:207-219.

32. Duesberg, P.H., K. Bister, C. Moscovici. 1980. Genetic structure of avian myeloblastosis virus, released from transformed myeloblasts as a defective virus

particle.

Proc. Nat. Acad. Sci. 77:5120-5124.

33. Eisenman, R., R. Shaikh, and W.S. Mason. 1978. Identification of an avian oncovirus polyprotein in uninfected chick cells. Cell 14:89-104.

34. Eisenman, R.N., W.S. Mason, and M. Linial. 1980. Synthesis and processing of polymerase proteins of wild type and mutant avain retroviruses. J. Virol. 36:63-782-78.

35. Erikson, E., M.S. Collett, and R.L. Erikson. 1978. In vitro synthesis of a functional avian sarcoma virus transforming gene product. Nature (London) 274:919-921.

36. Erikson, R.L., M.S. Collett, E. Erikson, and A.F. Purchio. 1979. Evidence that the avian sarcoma virus transforming gene product is a cyclic AMP independent protein kinase. Proc. Natl. Acad. Sci. USA 76:6260-6264.

37. Gilboa, E., S.W. Mitra, S. Goff, and D. Baltimore. 1979. A detailed model of reverse transcription and tests of crucial aspects. Cell 18:93-100.

38. Gilmer, T., and J.T. Parsons. 1979. Analysis of cellular integration sites in avian sarcoma virus-infected duck embryo cells. J. Virol. 32:762-769.

39. Groner, B., and N.E. Hynes. 1980. Number and location of mouse mammary tumor virus proviral DNA in mouse DNA of normal tissue and of mammary tumors. J. Virol. 33:1013-1025.

40. Gross, L. 1970. Oncogenic viruses. 2nd edition. Pergamon Press (New York).

41. Hanafusa, H. 1964. Nature of the defectiveness of Rous sarcoma virus. Natl. Cancer Inst. Monogr. 17:543-556.

42. Hanafusa, H. 1969. Rapid transformation of cells by RSV. Proc. Natl. Acad. Sci. USA 63:318-325.

43. Hanafusa, H., C.C. Halpern, D.L. Buchagen, and S. Kawai. 1977. Recovery of avian sarcoma virus from tumors induced by transformation-defective mutants. J. Exp. Med. 146:1735-1747.

44. Hanafusa, H., D. Baltimore, D. Smoler, K.F. Watson, A. Yaniv, and S. Spiegelman. 1972. Absence of polymerase protein in virions of alpha-type Rous sarcoma virus. Science 177:1188-1191.

45. Hanafusa, H., T. Hanafusa, and H. Rubin. 1963. The defectiveness of Rous sarcoma virus. Proc. Natl. Acad. Sci. USA 49:572-580.

46. Hanafusa, H., and T. Hanafusa. 1968. Further studies on RSV production from transformed cells. Virlogy

34:630-636.

47. Hanafusa, H., and T. Hanafusa. 1971. Non-infectious RSV deficient in DNA polymerase. Virology 43:313-316.

48. Hanafusa, T., H. Hanafusa, and T. Miyamoto. 1970. Recovery of a new virus from apparently normal chick cells by infection with avian tumor viruses. Proc. Natl. Acad. Sci. USA. 67:1797-1800.

49. Harvey, J.J. 1964. An unidentified virus which causes the rapid production of tumors in mice. Nature (London) 204:1104-1105

50. Haseltine, W.A., A.M. Maxam, and W. Gilbert. 1977. Rous sarcoma virus genome is terminally redundant: The 5' sequence. Proc. Natl. Acad. Sci. USA 74:989-993.

51. Hayman, M.J. 1978. Viral polyproteins in chick embryo fibroblasts infected with avian sarcoma leukosis viruses. Virology 85:241-252.

52. Hayward, W.S. and H. Hanafusa. 1975. Recombination between endogenous and exogenous RNA tumor virus genes as analyzed by nucleic acid hybridization. J. Virol. 15:1367-1377.

53. Hayward, W.S., S.B. Braverman, and S.M. Astrin. 1980. Transcriptional products and DNA structure of endogenous avian proviruses. Cold Spring Harbor Symp. Quant. Biol. 44:1111-1121.

54. Highfield, P.H., L.F. Rafield, T.M. Gilmer, and J.T. Parsons. 1980. Molecular cloning of avian sarcoma virus closed circular DNA: Structural and biological characterization of three recombinant clones. J. Virol. 36:271:279.

55. Hill, M. and J. Hillova. 1972. Virus recovery in chicken cells tested with Rous sarcoma cell DNA. Nat. New Biol. 237:35-39.

56. Hishinuma, F., P.J. DeBona, S. Astrin, and A.M. Skalka. 1981. Nucleotide sequence of acceptor site and termini of integrated avian endogenous provirus evl: Integration creates a 6 bp repeat of host DNA. Cell 23:155-164.

57. Hohn, B. and K. Murray. 1977. Packaging of recombinant DNA molecules into bacteriophage particles in vitro. Proc. Natl. Acad. Sci. USA 74: 3259-3263.

58. Hsu, T.W., J.L. Sabran, G.E. Mark, R.V. Guntaka, and J.M. Taylor. 1978. Analysis of unintegrated avian RNA tumor virus double-stranded DNA intermediates. J. Virol. 28:810-818.

59. Hughes, S.H. 1982. Sequence of the long terminal repeat and adjacent segments of the endogenous avian virus Rous associated virus 0. J. Virol. 43:191-200.

60. Hughes, S.H., K. Toyshima, J.M. Bishop, and H.E. Varmus.

1981. Organization of the endogenous proviruses of chickens: Implications for origin and expression. Virology 108:189-207.

61. Hughes, S.H., P.K. Vogt, E. Stubblefield, J.M. Bishop, and H.E. Varmus. 1981. Integration of avian sarcoma virus DNA in chicken cells. Virology 108:208-221.

62. Hughes, S.H., P.R. Shank, D.H. Spector, H-J Kung, J.M. Bishop, H.E. Varmus, P.K. Vogt, and M.L. Breitman. 1978. Proviruses of avian sarcoma virus are terminally redundant, co-extensive with unintegrated linear DNA, and integrated at many sites. Cell 15:1397-1410.

63. Hughes, S.R., A. Mutschler, J.M. Bishop, and H.E. Varmus. 1981. A Rous sarcoma virus provirus is flanked by short direct repeats of a cellular DNA sequence present in only one copy prior to integration. Proc. Natl. Acad. Sci. USA 78:4299-4303.

64. Jenkins, N.A., and G.M. Cooper. 1980. Integration, expression, and infectivity of exogenously acquired proviruses of Rous associated virus-0. J. Virol. 36:684-691.

65. Ju, G., L. Boone, and A.M. Skalka. 1980. Isolation and characterization of recombinant DNA clones of avian retroviruses: Size heterogeneity and instability of the direct repeat. J. Virol. 33:1026-1033.

66. Ju, G., and A.M. Skalka. 1980. Nucleotide sequence analysis of the long terminal repeat (LTR) of avian retroviruses: structural similarities with transposable elements. Cell 22:379-386.

67. Junghans, R.P., L.R. Boone, and A.M. Skalka. 1982. Products of reverse transcription in avian retrovirus analyzed by electron microscopy. J. Virol. 43:544-554.

68. Karess, R.E. and H. Hanafusa. 1981. Viral and cellular src genes contribute tohe structure of recovered avian sarcoma virus transformed protein. 24:155-164.

69. Kawai, S. 1980. Transformation of rat cells by fusion-infection with Rous sarcoma virus. J. Virol. 34:772-776.

70. Kawai, S. and H. Hanafusa. 1972. Genetic recombinaton with avian tumor virus. Virology 49:37-44.

71. Kawai, S. and H. Hanafusa. 1973. Isolation of defective mutant of avian sarcoma virus. Proc. Natl. Acad. Sci. USA 70:3493-3497.

72. Kawai, S. and H. Hanafusa. 1971. The effects of reciprocal changes in temperature on the transformed state of cells infected with a Rous sarcoma virus mutant. Virology 46:470-479.

73. Kawai, S., P.H. Duesberg, and H. Hanafusa. 1977.

Transformation defective mutants of Rous sarcoma virus with src gene deletions of varying lengths. J. Virol. 24:910-914.

74. Keshet, E., and H.M. Temin. 1978. Sites of integration of reticuloendotheliosis virus DNA in chicken DNA. Proc. Natl. Acad. Sci. USA 75:3372-3376.

75. Kessler, S.W. 1975. Rapid isolation of antigens from cells with a Staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein. Am. J. Immunol. 115:1671-1624.

76. Ketner, G., and T.J. Kelly. 1976. Integrated SV40 sequences in transformed cell DNA: Analysis using restriction endonucleases. Proc. Natl. Acad. Sci. USA 73:1102-1106.

77. Kitamura, N., A. Kitamura, K. Toyoshima, Y. Hirayama, and M. Yoshida. 1982. Avian sarcoma virus Y73 genome sequence and structural similarity of its transforming gene product to that of Rous sarcoma virus. Nature (London) 297:205-208.

78. Klempnaueer, T.J. Gonda, and J. M. Bishop. 1982. Nucleotide sequence of the retroviral leukemia gene v-myb and its cellular progenitor c-myb: the architecture of a transduced oncogene. Cell 31:453-463.

79. Kung, H.J., Y.K. Fung, J.E. Majors, J.M. Bishop, and H.E. Varmus. 1981. Synthesis of plus strands of retroviral DNA in cells infected with avian sarcoma virus and mouse mammary tumor virus. J. Virol. 37:127-138.

80. Laemmli, U.K. 1970. Clavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

81. Landy, A., and W. Ross. 1977. Viral integration and excision: Structure of lambda att sites. Science 197:1147-1160.

82. Leder, P., Temier, D. and L. Enquist. 1977. EK2 derivatives of bacteriophage lambda useful in the cloning of DNA from higher organisms: the lambda gtWES system. Science 196: 175-177.

83. Lerner, R.M., J.A. Boyle, S.M. Mount, S.L. Wolin, and J.A. Steitz. 1980. Are snRNPs involved in splicing? Nature (London) 283:220-224.

84. Linial, M. and D. Blair. 1982. Genetics of retroviruses. In RNA Tumor Viruses, Weiss, R., N. Teich, H. Varmus, and J. Coffin, eds. Cold Spring Harbor Laboratory (New York) pp. 698-702.

85. Linial, M., S. Brown, and P. Neiman. 1978. A noncondi-
tional mutant of Rous sarcoma virus containing defec-

tive polymerase. Virology 87:130-141.

86. Majors, J.E., and H.E. Varmus. 1981. Nucleotide sequences at host-proviral junctions for mouse mammary tumor virus. Nature (London) 289:253-258.

87. Mandel, M. and A. Higa. 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. 53:159

88. Maniatis, T., A. Jeffrey, and D.G. Kleid. 1975. Nucleotide sequence of the rightward operator of phage. Proc. Natl. Acad. Sci. USA 72:1184-1188.

89. Martin, G.S. 1970. Rous sarcoma virus: a function required for the maintenance of the transformed state. Nature (London) 227:1021-1023.

90. Mason, W.S., T.W. Hsu, C. Yeater, J.L. Sabran, G.E. Mark, A. Kaji, and J.M. Taylor. 1979. Avian sarcoma virus transformed quail clones defective in the production of focus-forming units. J. Virol. 30:132-140.

91. Mathews, R.E.F. 1979. Classificaton and nomenclature of viruses. Intervirology. 12:234-238.

92. Maxam, A.M. and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Meth. Enzymol. 65:499-560.

93. Mitra, S.W., S. Goff, E. Gilboa, and D. Baltimore.

1979.

Synthesis of a 600-nucleotide-long plus strand DNA by virions of Moloney murine leukemia virus. Proc. Natl. Acad. Sci. USA 76:4355-4359.

94. Moelling, K., A. Scott, K.E.J. Dittmar, and M. Owada. 1980. Effect of p15-associated protease from an avian RNA tumor virus on avian virus-specific polyprotein precursors. J. Virol. 33:680-688.

95. Morgan, H.R. and W. Traub. 1964. Origin of Rous sarcoma virus strains. Natl. Cancer Inst. Monogr. 17:392-393.

96. Murray, N.E., Brammer, W.J. and K. Murray. 1977. Lamboid phages that simplify the recovery of in vitro recombinants. Mol. Gen. Genet. 150: 53-61

97. Neel, B.G., W.S. Hayward, H.L. Robinson, J. Fang, and S.M. Astrin. 1981. Avian leukosis virus-induced tumors have common proviral integration sites and synthesize discrete new RNAs: Oncogenesis by promoter insertion. Cell 23:323-334.

98. O'Rear, J.J. and H.M. Temin. 1981. Mapping of alterations in noninfectious proviruses of spleen encrosis virus. J. Virol. 39:138-149.

99. O'Rear, J.J., S. Mizutani, G. Hoffman, M. Fiandt, and H.M. Temin. 1980. Infectious and non infectious

recombinant clones of the provirus of SNV differ in cellular DNA and are apparently the same in viral DNA. Cell 20:423-430.

100. Oppermann, H, J.M. Bishop, H.E. Varmus, and L. Levintow. 1977. A joint product of the genes gag and pol of avian sarcoma virus: a possible precursor of reverse transcriptase. Cell 12:993-1005.

101. Panet, A., D. Baltimore, and T. Hanafusa. 1975. Quantitation of avian RNA tumor virus reverse transcriptase by radioimmunoassay. J. Virol. 16:146-152.

102. Panet, A., W.A. Haseltine, D. Baltimore, G. Peters, F. Harada, J.E. Dahlberg. 1975. Specific binding of tryptophan transfer RNA to avian myeloblastosis virus RNA-dependent DNA polymerase (reverse transcriptase). Proc. Natl. Acad. Sci. USA 72:2535-2539.

103. Pepinsky, R.B. and V.M. Vogt. 1983. Purification and properties of a fifth major viral gag protein from avian sarcoma and leukemia viruses. J. Virol. 45:648-658.

104. Peters, G.G., and J. Hu. 1980. Reverse transcriptase as the major determinant for selective packaging of tRNA into avian sarcoma virus particles. J. Virol. 36:692-700.

105. Rettenmeir, C.W., R.E. Karess, S.M. Anderson, and H.

Hanafusa.

1979. Tryptic peptide analysis of avian oncovirus gag and pol gene products. J. Virol. 32:102-113.

106. Rettenmier, C.W., and H. Hanafusa. 1977. Structural protein markers in the avian oncoviruses. J. Virol. 24:850-864.

107. Rous, P. 1911. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. J. Exp. Med. 13:397-411.

108. Rubin, C.M. and C.W. Schmid. 1980. Pyrimidine-specific chemical reactions useful for DNA sequencing. Nucl. Acids Res. 8:4613-4619.

109. Rubin, H. and P.K. Vogt. 1962. An avian leukosis virus associated with stocks of Rous sarcoma virus. Virology 17:184-194.

110. Rushlow K.E., J.A. Lautenberger, T.S. Papas, M.A. Baluda, B. Perbal, J.G. Chirikjian, E.P. Reddy. 1982. Nucleotide sequence of the transforming gene of avian myeloblastosis virus. Science 216:1421-1423.

111. Sabran, J.L., T.W. Hsu, C. Yeater, A. Kaji, W.S. Mason, and J.M. Taylor. 1979. Analysis of integrated avian RNA tumor virus DNA in transformed chicken, duck, and quail fibroblasts. J. Virol. 29:170-178.

112. Sawyer, R.C., C.W. Rettenmeir, and H. Hanafusa. 1979.

Formation of Rous associated virus-60: origin of the polymerase gene. J. Virol. 29:856-862.

113. Sawyer, R.C., and H. Hanafusa. 1979. Comparison of the small RNAs of polymerase-deficient and polymerase-positive Rous sarcoma virus and another species of avian retrovirus. J. Virol. 29:863-871.

114. Scheele, C.M. and H. Hanafusa. 1971. Proteins of helper-dependent RSV. Virology 45:401-410.

115. Schiff, R.D. and D.P. Grandgenett. 1978. Vius-coded origin of a 32,000 dalton protein from avian retrovirus cores: structural relatedness of p32 and the beta polypeptide of avian retrovirus DNA polymerase. J. Virol. 28:279-291.

116. Schnieke, A., H. Stuhlmann, K. Harbers, I. Chumakov, and R. Jaenisch. 1983. Endogenous Moloney leukemia virus in nonviremic mov substrains of mice carries defects in the proviral genome. J. Virol 45:505-513.

117. Schwartz, D.E., R. Tizard, and W. Gilbert. 1983. Nucleotide sequence of Rous sarcoma virus. Cell 32:853-869.

118. Shank, P.R., S.H. Hughes, H-J Kung, J.E. Majors, N. Quintrell, R.V. Guntaka, J.M. Bishop, and H.E. Varmus. 1978. Mapping unintegrated avian sarcoma virus DNA: Termini of linear DNA bear 300 nucleotides

present once or twice in two species of circular DNA. Cell 15:1383-1395.

119. Shank, P.R., S.H. Hughes, and H.E. Varmus. 1981. Restriction endonuclease mapping of the DNA of Rous associated virus-0 reveals extensive homology in structure and sequence with avian sarcoma virus DNA. Virology 108:177-188.

120. Shibuya, M. and H. Hanfusa. 1982. Nucleotide sequence of Fujinami sarcoma virus: evolutionary relationship of its transforming gene with transforming genes of other sarcoma viruses. Cell 30:787-795.

121. Shimotohno, K., S. Mizutani, and H.M. Temin. 1980. Sequence of retrovirus provirus resembles that of bacterial transposable elements. Nature (London) 285:550-554.

122. Shimotohno, K., and H.M. Temin. 1980. No apparent nucleotide sequence specifity in cellular DNA juxtaposed to retrovirus proviruses. Proc. Natl. Acad. Sci. USA 77:7357-7361.

123. Shinnik, T., R. Lerner, and J. Sutcliffe. 1981. Nucleotide sequence of Moloney leukemia virus. Nature (London) 293:543-548.

124. Shoemaker, C., S. Goff, E. Gilboa, M. Paskind, S.W. Mitra, and D. Baltimore. 1980. Structure of a cloned

circular Moloney murine leukemia virus DNA molecule containing an inverted segment: implications for retrovirus integration. Proc. Natl. Acad. Sci. USA 77:3932-3936.

125. Skalka, A.M., P. DeBona, F. Hishinuma, W. McClements. 1980. Avian endogenous proviral DNA: analysis of integrated ev-1 and a related gs^- chf^- provirus purified by molecular cloning. Cold Spring Harbor Symp. Quant. Biol. 44:1097-1104.

126. Sorge, J. and S.H. Hughes. 1982. Polypurine tract adjacent to the U_3 region of the Rous sarcoma virus genome provides a cis-acting function. J. Virol. 43:482-488.

127. Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 998:503-517.

128. Staden, R. 1977. Sequence data handling by computer. Nucl. Acids Res. 4: 4037-4051.

129. Steffen, D., and R.A. Weinberg. 1978. The integrated genome of murine leukemia virus. Cell 15:1003-1010.

130. Stehlin, D., H.E. Varmus, J.M. Bishop, and P.K. Vogt. 1976. DNA related to the transforming genes of avian sarcoma viuses is present in normal avian DNA. Nature (London) 260: 170-173.

131. Stephenson, J.R. and S.A. Aaronson. 1973. Characterization of temperature sensitive mutants of murine leukemia virus. Virology 54:53-59.

132. Swanstrom, R., J.M. Bishop, and H.E. Varmus. 1982. Structure of a replication intermediate in the synthesis of Rous sarcoma virus DNA in vitro. J. Virol. 42:337-341.

133. Takeya, T, H. Hanafusa, R.P. Junghans, G. Ju, and A.M. Skalka. 1981. Comparison between the viral transforming gene (src) of recovered avian sarcoma virus and its cellular homolog. Mol. Cell. Biol. 1:1024-1037.

134. Takeya, T. and H. Hanafusa. 1982. DNA sequence of the viral and cellular src gene of chickens. II. Comparison of the src genes of two strains of avian sarcoma virus and of the cellular homolog. J. Virol. 44:12-18.

135. Takeya, T. and H. Hanafusa. 1983. Structure and sequence of the celular gene homologous to the RSV src gene and the mechanism for the generating the transforming virus. Cell 32:881-890.

136. Takeya, T., R.A. Feldman, and H. Hanafusa. 1982. DNA sequence of the viral and cellular src gene of chickens. I. Complete nucleotide sequence of an Eco RI fragment of recovered avian sarcoma virus which codes

for gp37 and pp60-src. J. Virol. 44:1-11.

137. Taylor, J.M. and T.W. Hsu. 1980. Reverse transcription of avian sarcoma virus RNA into DNA might involve copying of the tRNA primer. J. Virol. 33:531-534.

138. Taylor, J.M., T.W. Hsu, C. Yeater, and W.S. Mason. 1980. Synthesis and integration of avian sarcoma virus DNA. Cold Spring Harbor Symp. Quant. Biol. 44:1091-1096.

139. Taylor, J.M., T.W. Hsu, and M.M.C. Lai. 1978. Restriction enzyme sites on the avian RNA tumor virus genome. J. Virol. 26:479-484.

140. Temin, H.M. and H. Rubin. 1958. Characteristics of an assay for Rous sarcoma virus and Rous sarcoma cells in culture. Virology 16:669-688.

141. Thurzo, V., J. Smida, V. Smidova-Kovarova, and D. Simkovic. 1963. Some properties of the fowl virus tumor B77. Acta Unio Int. Contra. Cancerum 19:304-305.

142. Verma, I.M. 1977. The reverse transcriptase. Biochim. Biophys. Acta 473:1-38.

143. Vogt, V.M., W. Wight, and R. Eisenman. 1979. In vitro cleavage of avian retrovirus gag proteins by viral protease p15. Virology 98:154-167.

144. Von der Helm, K. 1977. Cleavage of Rous sarcoma viral polyprotein precursor into internal structural pro-

teins in vitro involves viral protein p15. Proc. Natl. Acad. Sci. 74:911-915

145. Wang, L-H and P.H. Duesberg. 1973. DNA polymerase of muring sarcoma-leukemia virus: lack of detectable RNase H and low activity with viral RNA and natural DNA templates. J. Virol. 12:1512-1521.

146. Wang, L-H, C.C. Halpern, M. Nadel, and H. Hanafusa. 1978. Recombination between viral and cellular sequences generates transforming ssarcoma virus. Proc. Natl. Acad. Sci. USA 75:5812-5816.

147. Weiss, S.R., P.B. Hackett, H. Oppermann, A. Ullrich, L. Levintow, and J.M. Bishop. 1978. Cell-free translation of avian sarcoma virus RNA: Suppression of the gag termination codon does not augment synthesis of the joint gag-pol product. Cell 15:607-614.

148. Weissbach, A., A. Bolden, R. Muller, H. Hanafusa, and T. Hanafusa. 1972. Deoxyribonucleic acid polymerase activities in normal and leukosis-infected chicken embryo cells. J. Virol. 10:321-327.

149. Witte, O.N., and D. Baltimore. 1978. Relationship of retrovirus polyprotein cleavages to virion maturation studied with temperature-sensitive murin leukemia virus mutants. J. Virol. 26:750-761.

150. Wong, P.K.Y., L.J. Russ, and J.A. McCarter. 1973. Ra-

pid, selective procedure for isolation of spontaneous temperature-sensitive mutants of Moloney leukemia virus. Virology 51:424-431.

151. Yamamoto, T., G. Jay, and I. Pastan. 1980. Unusual features in the nucleotide sequence of a cDNA clone derived from the common region of avian sarcoma virus messenger RNA. Proc. Natl. Acad. Sci. USA 77:176-180.

152. Yamamoto, T., B. deCrombrugghe, and I. Pastan. 1980. Identification of a functional promoter in the long terminal repeat of Rous sarcoma virus. Cell 22:787-797.

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