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John C. Minnerly

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STUDIES OF THE DNA TRANSCRIPTS SYNTHESIZED IN A RECONSTRUCTED REACTION WITH AMV 35S RNA AND PURIFIED REVERSE TRANSCRIPTASE

By
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B.A., University of Montana, 1980
Presented in partial fulfillment of the requirements for the degree of Master of Science UNIVERSITY OF MONTANA 1984

Approved by:

Kenneth J. Watson,
Chairman, Board of Examiners

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Date 3-1-84
The avian myeloblastosis virus (AMV) reconstructed reaction was used to investigate the DNA transcripts synthesized with AMV 35S RNA and purified RNA-directed DNA polymerase (reverse transcriptase). The goals of this study were: 1) to optimize in the presence of a new buffer system the reconstructed reaction with respect to genomic-length DNA synthesis and to examine the DNA transcripts synthesized; 2) to construct a restriction map of the DNA products based upon cDNA transcripts of various lengths; and 3) to attempt to detect whether genomic DNA transcripts synthesized in a reconstructed reaction contain a copy of the second long terminal redundancy (LTR).

Each component of the AMV reconstructed reaction was examined to ascertain its effects on genomic-length DNA synthesis. Use of potassium phosphate (pH 8.0) instead of Tris-HCl as a buffer resulted in enhanced synthesis of genomic DNA (7.8-8.0 kb) at a faster rate. In addition, several plus DNA species of discrete lengths were detected that are present in the Tris-buffered system.

Restriction mapping was performed using cDNA-plus DNA duplexes. Multiple restriction sites were located in the 5'-half of the cDNA template with the restriction enzymes Eco RI, Bam HI, Kpn I, Xho I, Hind III, and Hgi Al. With the exception of Hgi Al, the restriction sites detected were identical to those previously mapped in studies involving AMV-specific DNA isolated from infected cells. Hgi Al has not been used prior to this study to map AMV. Therefore, two Hgi Al-specific restriction sites located on the AMV genome are presented for the first time. The mapping method was simple, direct, and could possibly be applied to other viral and non-viral RNA species.

The second LTR (5' LTR) was not specifically detected. LTR-containing cDNA transcripts were detectable, although the procedure was not sufficiently selective to distinguish between cDNA transcripts containing either the first or second LTR.

These studies demonstrate that the optimized AMV phosphate-buffered reconstructed reaction is capable of synthesis of genomic-length DNA biochemically similar to those species found in the endogenous reaction or from infected cells. This suggests that the phosphate-buffered system may be useful in further detailed analysis of the events of retrovirus replication.
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ABBREVIATIONS

AMV        - avian myeloblastosis virus
Cr.        - the product of the concentration of ribonucleotides expressed in units of moles per liter times the time in seconds
ddH₂O       - double-distilled H₂O
cDNA       - DNA complementary to the RNA genome
plus DNA   - DNA anticomplementary to the RNA genome (same chemical polarity)
DTT        - dithiothreitol
EDTA       - ethylenediamine tetraacetic acid
hp DNA     - hairpin DNA
MAV        - myeloblastosis-associated virus
oligo(dT)  - deoxythymidylic acid
poly(rA)   - polyadenylic acid
SDS        - sodium dodecyl sulfate
Tris       - Tris (hydroxymethylaminomethane)
tRNA<sup>Trp</sup> - transfer RNA specific for the amino acid tryptophan
STR, R     - short terminal redundancy
LTR        - long terminal redundancy
U<sub>3</sub> - unique sequences corresponding to the 3'-end of the RNA genome
U<sub>5</sub> - unique sequences corresponding to the 5'-end of the RNA genome
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Kenneth F. Watson, for his understanding, patience, and guidance during these studies.

I am also grateful to my colleague, Dr. John M. Ong, for his helpful advice and our many discussions.

I would also like to thank my sister, Sue, for her assistance in typing this manuscript. And last, but certainly not least, I am grateful to Lucy for her encouragement and support in preparation of this thesis.
CHAPTER 1

INTRODUCTION

The first evidence of a link between some forms of cancer and sub-microscopic, filterable agents was reported over 75 years ago when Ellerman and Bang (1908) described the transmission of avian leukemia by a filterable agent. Later, Rous (1911) reported that chicken sarcomas could be induced by cell-free filtrates originating from a solid tumor. Yet these agents were largely ignored as experimental agents until recently (see Gross, 1970).

Today it is recognized that these agents were, in fact, viruses and they belong to the group of viruses known as retroviruses. Retroviruses, formally known as RNA tumor viruses, are a part of the family known as Retroviridae and belong to the subfamily Oncoviridae (Fenner, 1976).

All retroviruses share common characteristics: similar architecture, a set of structural proteins that are somewhat homologous; a genome consisting of a diploid dimer of single-stranded RNA; a virion polymerase capable of RNA-directed DNA synthesis (reverse transcription); and the presence of a double-stranded DNA intermediate during the viral replication cycle.

Avian Myeloblastosis Virus

One member of the family of Retroviridae, Avian Myeloblastosis Virus (AMV), consists of a complex of at least two types of retroviruses (Beard, 1963a). One of these, AMV proper, is replication defective, causes acute myeloblastic leukemia (AML) in chickens, and is able to transform myeloid
precursor cells in vitro (Beaudreau et al., 1960; Baluda et al., 1961; Beard, 1963a,b; Moscovici and Zanetti, 1970). In addition to AML, AMV also causes several types of neoplasia in chickens, including visceral lymphoid leukosis, nephroblastomas, and osteogenic osteoblastomas (Smith and Moscovici, 1969). Because AMV is replication defective, it is only infectious on the presence of a non-replication defective helper virus.

In addition to the leukemogenic agent responsible for myeloblastosis, the AMV complex is composed of another type of virus, consisting of two myeloblastosis-associated viruses known as MAV-1 and MAV-2 (Moscovici, 1975). MAV-1 and MAV-2 are replication competent, function as helper viruses for AMV, and also cause the three types of neoplasias mentioned previously (Smith and Moscovici, 1969; Moscovici, 1975). Unlike AMV, MAV-1 and MAV-2 cannot transform myeloid precursor cells in vitro. The mechanism by which helper viruses induce neoplasia differs from that used by AMV to induce acute myeloblastic leukemia in that oncogenesis by AMV appears to be direct (Baluda et al., 1983). MAV-1 and MAV-2 lack the gene carried by AMV which induces neoplasia, and oncogenesis by these helper viruses occurs indirectly, presumably by viral activation of a cellular gene having oncogenic potential (Baluda et al., 1983).

Structurally, AMV is typical of most retroviruses in that a centrally located, spherical core structure containing nucleic acid and core protein is surrounded by an outer lipid membrane (Bonar et al., 1963; Luftig et al., 1974). Data from both electron microscopy and hydrodynamic studies show that AMV has a diameter of 140 nm (Luftig et al., 1974; Salmeeen et al., 1975). Dry weight analysis of AMV reveals that 65% of the total mass of AMV is present as protein (Bonar and Beard, 1959) with 5-7% of the
total protein present consisting of glycoprotein. The majority of the rest of the mass of AMV is lipid and phospholipid, which comprises 30-35% of the total weight (Bonar and Beard, 1959; Quigley et al., 1971, 1972). Only 2% of the total mass of AMV is RNA and trace amounts of DNA are also present (Levinson et al., 1972).

The AMV Genome

The genome of the AMV complex is a 60-70S RNA molecule composed of two identical 34-35S RNA subunits 7.2-7.8 kb in length (Billeter et al., 1974; Delius et al., 1974; Beemon et al., 1976; King, 1976; Kung et al., 1976; Duesberg et al., 1980; Chen et al., 1981; Gonda et al., 1981). Although the genome lengths of MAV-1 and MAV-2 are equivalent in size (7.5-7.8 kb), the size of the AMV proper RNA genome is smaller (7.2-7.5 kb) in length (Chen et al., 1981; Gonda et al., 1981). The 34-35S RNA subunits are linked to each other near their respective 5'-termini (Bender and Davidson, 1976). The nature of the linkage has not yet been determined precisely, although agents that disrupt hydrogen bonding (heat, formamide, urea and dimethylsulfoxide) are able to dissociate the subunits (Duesberg, 1980).

The RNA genomes of both AMV and the helper viruses are structurally similar to eukaryotic messenger RNA. The 5-terminus is "capped" by the methylated cap structure $\text{m}^7\text{G}^\ast 5'\text{ppp}5'\text{Gm}$ (Furichi et al., 1975; Keith and Fraenkel-Conrat, 1975; Stoll et al., 1977). The majority of viral RNA subunits also contain a 100-200 nucleotide poly(rA) tail at the 3'-terminus (Stephenson et al., 1973; Wang and Duesberg, 1974). In addition, the viral RNA subunits also contain low levels of methylation (ten $N^6$-methyladenosine residues per subunit) at internal sites (Furichi et al., 1975;
Beemon and Keith, 1977; Dimock and Stoltfus, 1977). These three features are probably due to post-transcriptional modification of the viral RNA by host cell enzymes.

Another major viral related RNA species associated with AMV is a 21S RNA (2.3-2.5 kb) containing nucleotide sequences from both the 5- and 3'-termini of the 35S RNA genome (Chen et al., 1981; Gonda et al., 1981). The 21S RNA is believed to be a subgenomic mRNA which encodes the AMV transforming gene. Unlike most other retroviral subgenomic RNAs, the 21S RNA is present in significant amounts in the 60-70S RNA complex and is encapsidated in the virion (Stacey et al., 1977; Gonda et al., 1981). The appearance of 21S RNA is possibly due to a splicing mechanism involving the genomic 35S RNA.

Other nucleic acids associated with avian retroviruses include minor amounts of host cell 28S, 18S, and 5S ribosomal RNA (Obara et al., 1971; Faras et al., 1973), host transfer RNAs (tRNAs) (Sawyer and Dahlberg, 1973; Waters and Mullin, 1977), cellular derived polyribosomal 7S RNA (Erikson and Erikson, 1976), and small traces of DNA (Riman and Beaudreau, 1970; Levinson et al., 1972; Darlix et al., 1977). With the exception of the 28S and 18S rRNA, these nucleic acids are found only in minor amounts associated with the 60-70S RNA complex (Sawyer and Dahlberg, 1973; Faras et al., 1973; Darlix et al., 1977). Other than the tRNA specific for tryptophan (tRNA^Trp), which acts as a primer for DNA synthesis in AMV, it is unknown whether these extragenomic nucleic acids are randomly encapsidated in the AMV viral particle upon budding from the cell or if they serve an as yet unknown, specific purpose.
Structural Features of the 35S RNA Genome

AMV is similar to other retroviruses in that a RNA primer molecule is required to initiate synthesis of DNA. Specifically, the RNA primer specific for AMV is the transfer RNA specific for tryptophan. The tRNA$^{\text{Trp}}$ primer is located 100 nucleotides from the 5'-terminus of the 35S RNA subunit (Stoll et al., 1977) and has been shown to be specific for tryptophan as determined by its primary nucleotide sequence and by its amino acid accepting ability (Harada et al., 1975). Only the last 16 nucleotides on the 3' end of the tRNA$^{\text{Trp}}$ primer molecule are bound to the 35S RNA template.

Retroviruses are unusual in that the genomic RNA contains repeated sequences at the 5'- and 3'-termini of the RNA subunit (Haseltine et al., 1977; Schwartz et al., 1977; Stoll et al., 1977). With respect to AMV, this repeat sequence (known as the direct terminal or short terminal redundancy) is 19 nucleotides in length (Stoll et al., 1977) immediately adjacent to the 5' cap structure and either 16 or 19 nucleotides in length at the 3'-terminus adjacent to the poly(rA) tail. The heterogeneity in length of the short terminal redundancy (STR) at the 3'-terminus is due to the presence or absence of the trinucleotide sequence 5'-CCA-3' (Stoll et al., 1977). The origin of the heterogeneity at the 3'-terminus is unknown, but may reflect true genetic heterogeneity within AMV or the heterogeneity may occur during transcription, post-transcriptional processing, or by exonucleolytic damage. Supporting the non-heritable theory is the fact that a similar terminal heterogeneity has been found in Rous sarcoma virus (Schwartz et al., 1977).

Avian retroviruses contain four known genes (Bishop, 1975). Three of
the four genes are required for replication (Fig. 1) of the retrovirus and include; \textit{gag}, which encodes the viral core structural proteins; \textit{env}, which encodes the glycoproteins found in the viral envelope; and \textit{pol}, the gene which encodes the RNA-dependent DNA polymerase. The fourth gene, \textit{onc}, is the gene responsible for oncogenesis. The \textit{onc} gene confers upon retroviruses the ability to induce neoplasia (Graf and Berg, 1978; Duesberg, 1980; Sheiness et al., 1980). The term "onc" is a general one, and the identity of the \textit{onc} gene varies between different types of retroviruses. In the case of AMV, the \textit{onc} gene is known as \textit{amv} (previously known as \textit{myb} or \textit{luk}) and is the gene responsible for transformation of hematopoietic cells (Roussel et al., 1979; Chen et al., 1980; Souza et al., 1980; Baluda et al., 1983).

In contrast to many other retroviruses, neither the RNA genomes of AMV or MAV-1 and MAV-2 contain all four genes (Baluda et al., 1983). Both MAV-1 and MAV-2 encode for all three structural genes; \textit{gag}, \textit{env}, and \textit{pol}, but lack the leukemogenic sequences for the \textit{amv} gene. The 34S RNA genome of AMV lacks the \textit{env} gene in its entirety and probably contains a defective \textit{pol} gene (Souza et al., 1980; Baluda et al., 1983). The most likely explanation is that the \textit{amv} gene arises by cellular insertion. Evidence supporting this idea comes from the fact that cellular DNA sequences analogous to \textit{amv} have been found in chickens, ducks, human hematopoietic neoplastic cells, and other vertebrate species (Souza et al., 1980; Bergmann et al., 1981; Perbal and Baluda, 1982a,b; Westin et al., 1982; Baluda et al., 1983). Studies using leukemic chicken DNA have shown that the cellular \textit{amv} sequences (c-amv or proto-amv) are contained in a unique DNA segment 8-9 kb in length and are split by at least five large intervening
Fig. 1. Virus-Specific RNA Species Detected in AMV Transformed Myeloblasts. Three major virus-specific RNA species found in AMV transformed myeloblasts are presented with their relative sizes (Adapted from Chen et al., 1981).

- **35S RNA** - MAV-1 and MAV-2 RNA genome
- **34S RNA** - AMV RNA genome
- **21S RNA** - Subgenomic RNA species related to AMV 34S RNA

The abbreviations refer to the locations within each species of the gene coding sequences. See text for details.
sequences lacking homology with amv (Perbal and Baluda, 1982a,b; Baluda et al., 1983). It has been suggested that these cellular DNA sequences represent normal eukaryotic genes (Bishop et al., 1979; Duesberg, 1980; Bishop, 1981). Other cellular analogues homologous to the oncogenes of several other types of avian, feline, murine, and simian retroviruses have been discovered recently (Roussel et al., 1979; Spector et al., 1979; Stehelin et al., 1979; Canaani et al., 1980; Defeo et al., 1981; Shalloway et al., 1981; Vennstrom and Bishop, 1982; Vennstrom et al., 1982).

Another region of the 35S RNA genome is the c (common) region which is present in the first 400-500 nucleotides adjacent to the 3′-terminal poly(rA) tail (Wang, 1978). This region is similar in most, if not all, retroviruses, and is highly conserved (Tsichlis and Coffin, 1980). Sequencing studies of avian sarcoma virus RNA has shown that the c region contains numerous stop codons in all three reading frames, and is probably not translated (Czernilofsky et al., 1980; Yamamoto et al., 1980). The c region is found in both the AMV 34S RNA and MAV-1 and MAV-2 35S RNA genomes. The c region may serve a regulatory role since viral recombination studies suggest roles for this region in efficiency of virus replication, and possibly in oncogenesis by avian retroviruses lacking an onc gene (Tsichlis and Coffin, 1980; Robinson et al., 1980; Crittenden et al., 1981; Hayward et al., 1981; Neel et al., 1981).

Retrovirus Replication

In 1964, it was first proposed that a double-stranded DNA intermediate of the single-stranded RNA genome of retroviruses was required to induce transformation (Temin, 1964). In addition, to maintain the transformed state, the DNA intermediate was integrated into the host chromo-
somes in a mechanism similar to lysogenic bacteria. This provirus hypothesis of Temin has been supported by several major types of experiments. First, retroviral RNA genomes encode for the pol gene. All nondefective retroviruses contain the enzyme reverse transcriptase, which is capable of transcribing single-stranded RNA into double-stranded DNA (Green and Gerard, 1974; Verma, 1977). Second, the requirements for synthesis and integration of virus-specific double-stranded DNA in infected cells has been established by nucleic acid hybridization studies (Weinberg, 1977; Taylor, 1979). Last, transfection studies have demonstrated that the viral particles synthesized are infectious (Hill and Hillova, 1976).

**Reverse Transcriptase**

Reverse transcriptase as isolated from AMV-infected chicks has a molecular weight of 160,000-170,000 daltons and a sedimentation coefficient in glycerol gradients of 7.5S (Grandgenett et al., 1973; Varmus et al., 1977; Houts et al., 1979). It is a slightly acidic protein with an isoelectric point of 6.5 (Houts et al., 1979). As determined by SDS-polyacrylamide gel electrophoresis, AMV reverse transcriptase consists of two subunits, α and β, with apparent molecular weights of 65,000 and 95,000 daltons, respectively (Kacian et al., 1971; Grandgenett et al., 1973; Gibson and Verma, 1974). The smaller α subunit is structurally related to the β subunit as determined by two-dimensional fingerprint analysis (Gibson and Verma, 1974) and by comparison of the tryptic and chymotryptic maps of 125I-labelled α and β subunits of AMV reverse transcriptase (Rho et al., 1975). The structural relatedness of α to β suggests that α is a result of proteolytic cleavage of β. Like other DNA polymerases, AMV reverse transcriptase is a zinc metalloenzyme containing
1-2 g-atoms of bound zinc per mole of enzyme (Auld et al., 1974; Poiesz et al., 1974). Two primary enzymatic activities have been ascribed to AMV reverse transcriptase; a DNA polymerase which can transcribe either a single-stranded RNA or DNA template, and a ribonuclease (RNase H) specific for the RNA strand of a DNA-RNA hybrid (Moelling et al., 1971).

Phosphorylation and dephosphorylation of reverse transcriptase may constitute an important mechanism for its regulation. Reverse transcriptase activity is influenced by the extent of phosphorylation of the enzyme or enzyme-associated proteins isolated from AMV (Tsiapalis et al., 1976) and Rous sarcoma virus (Lee et al., 1975). In addition, purified reverse transcriptase buffered in Tris-HCl (pH 8.3) loses activity rapidly, while pools of reverse transcriptase buffered in potassium phosphate (pH 8.0) are relatively stable (Tsiapalis, 1976; unpublished observations). The loss of activity in Tris-buffered pools of reverse transcriptase could be completely restored by phosphorylation of the enzyme (Tsiapalis et al., 1976). The influence of phosphate observed in maintenance of reverse transcriptase activity is possibly due to its function as an endogenous phosphatase inhibitor (Strand et al., 1971; Silberstein et al., 1973).

Evidence in support of this theory comes from experiments demonstrating the deleterious effects of alkaline phosphatase on purified reverse transcriptase activity (Tsiapalis et al., 1976; unpublished results of Wolkowicz). In view of these findings, purified reverse transcriptase is buffered in potassium phosphate which prevents rapid loss of activity.

Reverse Transcription

The process of reverse transcription has been extensively studied using both infected cells and cell-free systems. Both approaches have
been invaluable in studying the mechanism of reverse transcription although each approach has its limitations.

Studies using infected cells are difficult because viral DNA comprises only 0.01% or less of the total cellular DNA population (Weinberg, 1977). Even with the application of sensitive procedures such as the blot-hybridization method of Southern (1975), it remains difficult to isolate viral DNA for detailed analysis. The techniques associated with recombinant DNA technology have been used with success to obtain genetic libraries of leukemogenic viral DNA clones (Silva et al., 1982). These clones have proven to be invaluable for investigation of the later stages of reverse transcription (Taylor, 1979). Studies using infected cells have another problem. It is difficult to define precisely the roles of viral and cellular enzymes involved in reverse transcription, especially event that occur in the early stages of reverse transcription.

In an attempt to avoid these problems, studies of the mechanism of reverse transcription have been performed using cell-free systems. One approach, the endogenous reaction, entails the use of nonionic detergent-disrupted virions in a suitable reaction mixture using the viral reverse transcriptase present in the virion (Baltimore, 1970; Temin and Mizutani, 1970). The endogenous reaction has been shown to be capable of transcribing single-stranded RNA into double-stranded DNA (Collett and Faras, 1975; Junghans et al., 1975; Rothenberg and Baltimore, 1976; Lai and Hu, 1978). The endogenous reaction has also been shown to be able to synthesize infective, genome-length double-stranded DNA copies of viral RNA (Rothenberg et al., 1977; Clayman et al., 1979; Gilboa et al., 1979; Boone and Skalka, 1980). One limitation of the endogenous reaction is that it is
difficult to precisely define the system in terms of the individual effect of each component. With a second approach, known as the reconstructed reaction, the process of reverse transcription is reconstructed by the addition of known, purified components, including viral genomic RNA and reverse transcriptase. This approach has been especially useful in examining the early events of reverse transcription.

Although the endogenous and reconstructed reactions are useful in studying the events of reverse transcription, they have similar limitations. First, the yield of genome-length DNA is relatively low (Taylor et al., 1978) in comparison with infected cell studies; and in early experiments only a limited representation of the viral RNA genome was transcribed into DNA (Garapin et al., 1973; Green and Gerard, 1976). Second, the total yield of DNA products from either reaction is small. Consequently, further detailed studies of the DNA products are difficult (Taylor et al., 1978). Recent studies have centered upon optimization of reaction conditions to enhance the yield of DNA in the reconstructed and endogenous reactions (Junghans et al., 1975; Rothenberg et al., 1977; Clayman et al., 1979; Gilboa et al., 1979; Boone and Skalka, 1980; Retzel et al., 1980; Schulz et al., 1981; Olsen, 1982). In the case of the endogenous reaction, it appears that all of the components necessary for reverse transcription are present within the virion, although the identities of the components necessary for efficient reverse transcription remain unknown. In contrast, the reconstructed reaction has not been shown to be capable of synthesis of complete, double-stranded DNA intermediates. This suggests that additional, unknown components found in the endogenous reaction are lacking in the reconstructed reaction.
Retrovirus DNA Synthesis in Cell-Free Systems

Studies using synthetic homopolymers have shown that the DNA polymerase activity associated with reverse transcriptase functions in an analogous manner as other DNA polymerases (Baltimore and Smoler, 1971; Goodman and Spiegelman, 1971; Leis and Hurwitz, 1972; Wells et al., 1972). The initial event that occurs is the covalent linking of a deoxyribonucleotide to the 3'-OH terminus of a RNA or DNA primer hydrogen-bonded to a DNA or RNA template. The primer molecule may be as short as 4-8 nucleotides (Baltimore and Smoler, 1971). Elongation of cDNA complementary to the template occurs in the 5' to 3' direction (Smoler et al., 1971). In the case for AMV, the tRNA Trp primer molecule is derived from the host chicken cell (Harada et al., 1975).

Each 35S RNA template contains only one tRNA Trp binding site (Sawyer and Dahlberg, 1973; Dahlberg et al., 1974) located 100 nucleotides from the 5'-terminus of the RNA template. Only nucleotides 2-17 from the 3'-end of the tRNA Trp primer are actually hydrogen-bonded to the RNA template (Cordell, 1976; Eiden et al., 1976).

Since the tRNA Trp primer is in close proximity to the 5'-terminus of the 35S RNA, the cDNA transcript only achieves a length of 100 nucleotides before the 5'-terminus of the RNA template is reached (see Fig. 2). This 100 nucleotide cDNA copy, known as "strong stop" cDNA or cDNA 100 5', is a major product of in vitro DNA synthesis under certain conditions (Friedrich et al., 1977; Haseltine et al., 1977; Stoll et al., 1977; Collett and Faras, 1978; Leis et al., 1978; Novak et al., 1979).

The question of how cDNA 100 5' elongates after it reaches the 5'-terminus of the RNA template has been a central issue (Taylor and Illmensee,
1975; Staskus et al., 1976; Collett and Faras, 1977; Haseltine et al., 1977; Taylor, 1977). Considerable evidence has accumulated supporting the idea of a transcriptional jump of cDNA\textsubscript{5\textsuperscript{100}} from the 5'-terminus of the 35S RNA to the 3'-terminus. It has been shown from pulse-chase studies and pyrimidine tract analysis that cDNA\textsubscript{5\textsuperscript{100}} is elongated (Haseltine et al., 1977; Rothenberg and Baltimore, 1977). Much of the RNA portion of the cDNA\textsubscript{5\textsuperscript{100}} is hydrolyzed, resulting in a single-stranded DNA tail (Darlix et al., 1977; Collett et al., 1978a; Friedrich and Moelling, 1979). In addition, there is the 16-19 nucleotide sequence at the 3'-end of the nascent cDNA\textsubscript{5\textsuperscript{100}} which is complementary to the short terminal redundancy adjacent to the poly(rA) tail at the 3'-terminus of the RNA template (Haseltine et al., 1977; Schwartz et al., 1977; Stoll et al., 1977). Sequencing studies of the cDNA products synthesized in the avian sarcoma virus endogenous and reconstructed reactions support the concept of a transcriptional jump (Omer et al., 1981; Swanstrom et al., 1981b).

DNA products have been synthesized in endogenous reactions using murine and avian retroviruses that approach a similar size and structure to some of the DNA intermediates found in infected cells. Detergent disrupted virions from murine leukemia virus (Gilboa et al., 1979a; Bosselman and Verma, 1980) or murine sarcoma virus (Benz and Dina, 1979) are capable of synthesizing full-length, double-stranded linear DNA. Restriction mapping studies of the linear duplex DNA have shown that it is indistinguishable from those found in the cytoplasm of infected cells, and that it does contain the long terminal repeat (Gilboa et al., 1979a,b). The cDNA is intact and contains the long terminal repeat (Boone and Skalka, 1981b). In contrast to the DNA products from the endogenous reac-
tion of murine retroviruses, the second strand of DNA (plus DNA) is frag-
mented (Clayman et al., 1979; Boone and Skalka, 1980,1981a,b). This re-
result is not surprising as the plus DNA of the linear form found in the
cytoplasm of avian retrovirus-infected cells is also fragmented, in con-
trast to the intact plus DNA found in murine retrovirus-infected cells
(Kung et al., 1981).

Another form of double-stranded DNA found in the murine (Gilboa et
al., 1979a; Dina and Benz, 1980) and avian sarcoma (Clayman et al., 1979;
Guntaka et al., 1980) endogenous reaction products is a covalently closed
circular form. Restriction mapping of this form from either the murine
leukemia virus (Gilboa et al., 1979a) or murine sarcoma virus (Dina and
Benz, 1980) systems have shown that it contains only one copy of the LTR
and resembles one of the two circular forms found in the nucleus of in-
fected cells. The circular forms from the avian sarcoma virus endogenous
reaction have not yet been mapped.

Synthesis of plus DNA is initiated before the completion of cDNA
synthesis in the endogenous reaction (Gilboa et al., 1979a; Dina and
Benz, 1980; Boone and Skalka, 1981a). The initial species of plus DNA,
at least in the case for the murine retrovirus endogenous reaction, is a
discrete fragment of uniform size (600 nucleotides) which appears very
early in the reaction. This early species of plus DNA has sequences ho-
mologous to sequences from the 5’- and 3’-ends of the viral RNA genome
(Mitra et al., 1979; Dina and Benz, 1980). Similar species of plus DNA
are synthesized early in infection by avian sarcoma virus (Varmus et al.,
1978; Kung et al., 1981) or mouse mammary tumor virus (Kung et al., 1981).

Many investigators have examined the DNA products from reconstructed
reactions using purified reverse transcriptase and the 60-70S RNA complex or the 35S RNA·tRNA subunits (Taylor et al., 1972; Leis and Hurwitz, 1972; Haseltine and Baltimore, 1976; Haseltine et al., 1976; Collett and Faras, 1977; Darlix et al., 1977; Hizi et al., 1977; Schulz et al., 1981; Olsen and Watson, 1982). In general, early studies using the reconstructed reaction were not as successful as studies of the endogenous reaction. The DNA products from these early studies did not approach the size of the DNA products found in infected cells. The longest cDNA transcripts synthesized approached only half the length of the RNA genome when either the 60-70S RNA complex or the 35S RNA·tRNA subunit was used (Haseltine and Baltimore, 1976; Haseltine et al., 1976; Collett and Faras, 1977; Darlix et al., 1977; Hizi et al., 1977). These results suggest that additional, unknown factors are present in the virus that facilitate the elongation of cDNA since the majority of the cDNA transcripts are primed with the tRNA primer (Haseltine and Baltimore, 1976; Haseltine et al., 1976), and purified reverse transcriptase alone is sufficient to make the correct transcriptional jump (Swanson et al., 1981). Later studies using AMV 35S RNA and purified reverse transcriptase in a reconstructed reaction have been more successful in synthesizing long copies of the RNA template using carefully optimized reaction conditions, but the total yield of genomic DNA remains small (Schulz et al., 1981; Olsen and Watson, 1982). Other studies using an oligo(dT) primer at the 3'-terminus of the RNA template have shown that the reconstructed reaction is capable of synthesis of nearly full-length cDNA copies in high yields (Myers and Spiegelman, 1977; Retzel et al., 1980).

Plus DNA has been reported to be synthesized early after the init-
iation of cDNA synthesis in the reconstructed reaction (Olsen and Watson, 1982). Other investigators have reported that purified reversed transcriptase is capable of transcribing the 60-70S viral RNA complex into double-stranded DNA (Taylor et al., 1972; Leis and Hurwitz, 1972; Hizi et al., 1977). In the reconstructed reaction using AMV reverse transcriptase and AMV 35S RNA•tRNA
tp, the plus DNA synthesized early in the reaction was found in two discrete sizes, 300 and 400 nucleotides in length (Olsen and Watson, 1982). Restriction mapping studies of these two species, known as plus DNA
\textsubscript{300} and plus DNA
\textsubscript{400}, suggested that they are related to each other and that they are located at or within the LTR region (Olsen and Watson, 1982). Plus DNA
\textsubscript{300} has sequences homologous to the 5'- and 3'-end sequences of plus DNA
\textsubscript{400}, and apparently contains an internal deletion of 100 nucleotides. Additional evidence for plus DNA
\textsubscript{300} and plus DNA
\textsubscript{400} comprising the LTR has come from nucleotide sequencing studies of AMV proviral DNA, which suggest that the LTR is composed of a 385 base pair sequence (Rushlow et al., 1982). Finally, cDNA probes specific for the LTR of AMV have detected plus DNA
\textsubscript{300} and plus DNA
\textsubscript{400} (Olsen and Watson, 1982). Other species of plus DNA unrelated to either plus DNA
\textsubscript{300} and plus DNA
\textsubscript{400} have also been detected in the AMV reconstructed reaction (Olsen and Watson, 1982). These species are relatively short in size (less than 1.5 kb) although genome-length cDNA was synthesized.

The Role of Ribonuclease H (RNase H)

In addition to its DNA polymerase activity, AMV reverse transcriptase has a second major enzymatic activity, ribonuclease H (Moelling et al., 1971). RNase H is RNase specific for the RNA moiety in a DNA•RNA hybrid (Moelling et al., 1971; Baltimore and Smoler, 1972; Keller and.
Crouch, 1972; Leis et al., 1973). The RNase H activity appears to be an intrinsic activity of reverse transcriptase (Verma, 1977) and probably has a different functional site that the DNA polymerase activity (Brewer and Wells, 1974; Verma, 1977; Lai and Verma, 1978).

In contrast to RNase H isolated from either calf thymus or Escherichia coli, AMV RNase H acts as a processive exonuclease (Grandgenett and Green, 1974; Verma, 1975) and requires free 3'-OH and 5'-PO₄ ends (Leis et al., 1973). Degradation of RNA is not dependent on concurrent cDNA synthesis since preformed DNA·RNA hybrids are susceptible to ribonuclease degradation (Baltimore and Smoler, 1972; Berma, 1975). The direction of hydrolysis of RNA by RNase H may be either in the 5' to 3' or the 3' to 5' direction (Leis et al., 1973). AMV RNase H shows some base specificity with respect to heteropolymeric DNA·RNA hybrids in that a majority of the hydrolysis products contain adenine at the 3'-terminus (Myers et al., 1980).

A salient point in reverse transcription concerns the role of RNase H. Available evidence points towards a dual role for RNase H: to help the cDNA₅₀ make the first transcriptional jump, and to provide a source of RNA primers for plus strand synthesis. Studies have shown limited and specific hydrolysis of the 5'-terminus of viral RNA shortly after initiation of cDNA synthesis (Darlix et al., 1977; Collett et al., 1978a; Friedrich and Moelling, 1979). Oligoribonucleotides about 12-15 nucleotides in length which contain the 5'-terminal cap structure are released from the template RNA after the completion of synthesis of cDNA₅₀ (Collett et al., 1978a,b). RNase H degradation products have been shown to function as primers for synthesis of plus DNA using homopolymeric tem-
Fig. 2. Summary of the Events of Reverse Transcription in Retroviruses.
Fig. 2. Summary of the Events of Reverse Transcription in Retroviruses.

A. AMV 35S RNA·tRNA\textsuperscript{Trp} genome. r - short terminal redundancy (str); p - 35S RNA primer binding site.

B. Initiation of cDNA synthesis. cDNA\textsuperscript{100} is synthesized in the 5'-3' direction to the RNA 5'-terminus, and is covalently linked to the tRNA\textsuperscript{Trp} primer. R - cDNA copy of the str.

C. RNase H hydrolysis of the 5'-terminus of the 35S RNA. The 35S RNA present in the form of a cDNA-RNA hybrid is hydrolyzed by RNase H.

D. First transcriptional jump. Base pairing of cDNA\textsuperscript{100} to the 35S RNA 3'-short terminal redundancy occurs.

E. Elongation of cDNA\textsuperscript{100}, and release of the intact poly(A) tail by RNase H.

F. RNase H hydrolysis of 35S RNA complementary to the nascent cDNA.

G. Initiation of plus DNA synthesis with residual 35S RNA fragments generated by RNase H hydrolysis of the cDNA-RNA hybrid. An early specie shown is plus DNA\textsuperscript{400}. R' - plus DNA copy of the str.

H. Strand displacement of 35S RNA template by plus DNA from the tRNA primer. Plus DNA synthesis is initiated at other sites and is discontinuous in AMV. At or near the same time, the nascent cDNA strand has copied the 35S primer binding site (p). P - cDNA copy of the 35S RNA primer binding site.

I. RNase H hydrolysis of the tRNA\textsuperscript{Trp} primer. It is not known if the primer molecule is hydrolyzed in its entirety or if the 5'-half is hydrolyzed separately (step H) as shown. P' - plus DNA copy of P.

J. Second transcriptional jump. The cDNA transcript hydrogen-bonded to the primer binding site displaces the original cDNA transcript.

K. Copying of plus DNA\textsuperscript{400} by the nascent cDNA strand, resulting in generation of the second LTR (5' LTR). The final form shown is the linear cytoplasmic form containing two copies of the LTR. The plus strand is shown fragmented. Ligation of the plus strand may be accomplished by host-specific ligases.
plate-primers (Watson et al., 1979) or AMV 35S RNA-oligo(dT) template-primer (Myers et al., 1980). Use of NaF in the homopolymeric template-primer system, which is a known inhibitor of RNase H, has been shown to inhibit synthesis of plus DNA (Watson et al., 1979).

Other enzymatic activities are associated with AMV (for a review, see Verma, 1977). In particular, a Mn^{++}-activated endonuclease capable of nicking covalently closed circular DNA but not linear DNA or single-stranded DNA or RNA has also been described (Golomb and Grandgenett, 1978). At present, the role of this endonuclease activity during reverse transcription is unknown.

An unwinding-like activity associated with reverse transcription which is capable of disrupting the hydrogen bonds of DNA-DNA duplexes has also been described (Collett et al., 1978b). It is not known if this unwinding activity resides with the reverse transcriptase molecule or with another protein. This activity has been associated with the removal of the 5'-terminal cap-containing oligoribonucleotide that is hydrolyzed from the cDNA_{5}^{100}·35S RNA hybrid prior to the first transcriptional jump (Collett and Faras, 1978).

Long Terminal Redundancy

The long terminal redundancy (LTR) flanks the double-stranded DNA replicative intermediate and is important in integration of the provirus and as a vehicle for control of viral RNA synthesis (Schwartz et al., 1977; Hughes et al., 1978). Unintegrated DNA in either the linear or covalently closed circular forms contains one or two copies of the LTR (Shank et al., 1978; Yoshimura and Weinberg, 1979; Shoemaker et al.,
1980; Van Beveren et al., 1980). The mechanism of integration of the provirus is not known precisely, but the LTR is structurally analogous to transposable elements (Sutcliffe et al., 1980; Swanstrom et al., 1981) and the provirus is integrated into multiple sites of the host chromosomes (Hughes et al., 1978).

LTRs contain sequences from both the 5' and 3'-termini of the viral RNA genome and are conveniently divided into three structural domains; the U₃, R, and U₅ regions, in respective order (Fig. 3). The U₃ region consists of sequences unique to the 3'-terminus of the viral RNA. This region shows the greatest size heterogeneity between viral species and accounts for much of the various lengths of the LTRs reported for different retroviruses (Temin, 1981). The relatively short R region (16-21 nucleotides) contains sequences from the short terminal redundancy of the viral RNA. The U₅ regions contains sequences unique to the 5'-terminus of the viral RNA. Both the R and U₅ regions of the AMV provirus are strongly conserved and show considerable homology with the R and U₅ regions of Rous sarcoma virus, Rous associated virus, and avian endogenous virus proviruses (Rushlow et al., 1982).

The size of the LTR varies widely between different retroviral species, ranging in size from 325 to 1300 nucleotides (Temin, 1981). The AMV LTR is 385 nucleotides in length (Rushlow et al., 1982) and is larger than the LTRs of many other avian retroviruses (Ju and Skalka, 1980; Yamamoto et al., 1980; Hishimuma et al., 1981; Swanstrom et al., 1981; Hughes, 1982) but considerably smaller than the LTRs of the mammalian Maloney sarcoma virus (Dhar et al., 1980) and spleen necrosis virus (Shimotohno et al., 1980)
Fig. 3. Structural Features of the AMV LTR.
Adapted from Rushlow et al., 1982. The 3' LTR is shown above. Sequences to the right of the AMV LTR are host cell sequences, while those to the left are AMV sequences.
The LTR of AMV is similar in structure and function to other retroviral LTRs and the sequences contain several notable features (Fig. 3). Each LTR is flanked by a 13 nucleotide inverted repeat with the sequence 5'-TGTAGTCTTAATC-3' (Rushlow et al., 1982). In unintegrated proviral DNA, the inverted repeat is two nucleotides longer. The two nucleotides are lost upon integration of the provirus (Temin, 1981). Twenty nucleotides downstream from the U3 inverted repeat is a tandem eleven nucleotide direct repeat sequence which is separated by an unrelated 15 nucleotide sequence. Tandem direct repeats and inverted repeats are characteristic of eukaryotic and prokaryotic transposable elements (Calso and Miller, 1980; Farabaugh and Fink, 1980; Gafun and Phillipsen, 1980). The direct repeats may also be important in initiation of transcription (Temin, 1981). The integrated provirus is also flanked by a duplicated six nucleotide host cell sequence, which is a common feature of prokaryotic (Bukhari et al., 1977) and eukaryotic (Dunsmuir et al., 1980; Farabaugh and Fink, 1980; Levis et al., 1980) transposable elements. Immediately upstream of the 3'-LTR is an eleven nucleotide polypurine sequence. This sequence has been observed in other retrovirus proviral DNA sequences and is believed to be a signal or initiation point for plus DNA synthesis (Swanstrom et al., 1981).

Several DNA sequences in the LTR are believed to play a role in the regulation of transcription. Immediately upstream from the R region are DNA sequences within the U3 region which have been implicated in transcriptional regulation. These sequences include a promoter-like sequence for initiation of transcription which is A-T rich (Pribnow, 1975) and a hexanucleotide sequence 5'-AATAAA-3' which is 20 nucleotides upstream.
from the poly(rA) acceptor sequence, 5'-CACA-3' (Proudfoot and Brownlee, 1976). Another sequence important for regulation of transcription is the tetranucleotide sequence 5'-GCCA-3' which is most likely the RNA capping site. Additional sequences, having the pentanucleotide sequence 5'-CCA-TT-3', are found 88 nucleotides upstream from the putative sequences coding for the 5'-cap structure. An analogous sequence appears 70-90 nucleotides upstream from the 5' end of the mRNA capping site in most eukaryotic structural genes (Efstratiadis et al., 1980). The presence of the "CAT" box sequences strengthens the argument that the LTR is involved in regulation of transcription. It has also been suggested that the regulatory sequences in the 3' LTR may also serve as promoters for mRNA extending into adjacent host sequences (Rushlow et al., 1982). Retroviral LTRs cloned in conjunction with eukaryotic and prokaryotic DNA serve as efficient promoters of RNA transcription (Gorman et al., 1982).

**Restriction Endonuclease Mapping of AMV**

Restriction mapping of DNA using type II restriction endonucleases has proven to be a useful analytical tool. Restriction endonucleases are enzymes that recognize and cleave specific DNA sequences. Restriction endonucleases have been used for isolation and purification of DNA, mapping of chromosomes and genes, recombination DNA experiments, and other types of studies (Nathans and Smith, 1975). Well over 200 different restriction enzymes have been purified and isolated.

Only recently has an extensive restriction map for the AMV complex (Fig. 4) been published (Bergmann et al., 1980, 1981; Olsen and Watson, 1982; Baluda et al., 1983). The AMV and MAV-1 proviruses share considerable sequence homology primarily in two regions of the viral RNA; a 5.5
Fig. 4. Restriction Map of the MAV-1 and AMV Provirus. Hatched box refers to location of the amv gene in the AMV provirus (adapted from Baluda, 1983).
kb fragment corresponding to the 5'-end of the viral RNA genome, and a 0.55 kb fragment corresponding to the 3'-terminus (Baluda et al., 1983). The heterogeneity in the restriction maps appears to be localized to a 1.0 kb region which in MAV-1 encodes the env gene and in AMV encodes for the amv gene (Baluda et al., 1983). This heterogeneity has been confirmed by heteroduplex mapping and R-loop mapping experiments (Souza et al., 1980). The MAV-2 provirus has not been as extensively mapped as MAV-1, but available data indicates that MAV-2 is closely related structurally to MAV-1 (Bergmann et al., 1980). MAV-2 contains four of the five Hind III restriction sites found in the MAV-1 provirus and both of the Eco RI sites.

The AMV LTR contains restriction sites for at least three restriction enzymes; Hind III (Bergmann et al., 1980, 1981), Hae III (Olsen and Watson, 1982), and Hpa I (Rushlow et al., 1982). Immediately upstream from the 3' LTR are single restriction sites for Xho I and Xba I (Baluda et al., 1983). These enzymes have proven useful in characterization of the LTR (Rushlow et al., 1982). Another useful restriction enzyme is Kpn I, which cleaves both the AMV and MAV-1 proviruses once, yielding two fragments of unequal size. The Kpn I cleavage site is located immediately upstream of the env gene in MAV-1 and the amv gene in AMV (Baluda et al., 1983).

Much of the restriction mapping data for the AMV complex has been performed using cloned proviral DNA from infected cells or from the endogenous reaction (Bergmann et al, 1980; Baluda et al., 1983). This is due primarily to the larger amount of proviral DNA that can be recovered from infected cells. However, restriction mapping using the DNA products synthesized in a reconstructed reaction would serve a dual purpose: 1) as
a confirmation of previously published results; and 2) to help demonstrate that the DNA products synthesized in a reconstructed reaction are essentially similar to those found in infected cells or from the endogenous reaction. This would suggest that the reconstructed reaction is capable of copying the RNA template with a high degree of fidelity. Development of a restriction map based on the DNA products synthesized in a reconstructed reaction could also be used to select specific cDNA probes which could be employed in detection of plus DNA synthesis.

**Proposal**

The reconstructed reaction is a system which contains purified reverse transcriptase and retroviral 60-70S RNA or 35S RNA subunits in a suitable reaction mixture which is capable of reverse transcription of the RNA template. Reconstructed reactions have been a useful approach to the study of the early events of reverse transcription. In this study, the AMV reconstructed reaction will be used to study retroviral RNA-directed DNA synthesis following the first transcriptional jump. To accomplish this, the AMV reconstructed reaction will be optimized further with respect to genomic-length DNA synthesis. Reaction conditions, including each component of the reaction mixture, will be varied to maximize synthesis of genomic-length DNA. A new buffering system, potassium phosphate (pH 8.0) will be employed as the buffer. Potassium phosphate may be a suitable buffer since reverse transcriptase is relatively stable in phosphate, and reverse transcriptase is believed to have a requirement for phosphorylation to maintain its activity. In addition to genomic-DNA synthesis, the DNA products synthesized in the phosphate-buffered reconstructed reaction will be examined for evidence of plus DNA synthesis.
Second, a restriction map based on the cDNA transcripts synthesized in the phosphate-buffered reconstructed reaction will be made. Mapping will be performed using presized cDNA transcripts that had been converted to double-stranded DNA with DNA primers and purified reverse transcriptase. Third, the genomic-length cDNA transcripts will be examined for evidence of sequences containing the second LTR using the technique of Southern blotting. Detection of the second LTR in genomic-length cDNA transcripts would strongly suggest that purified reverse transcriptase and 35S RNA-tRNA<sup>Trp</sup> alone are capable of making the second transcriptional jump.

Synthesis of DNA species in a reconstructed reaction indistinguishable from those found in vivo would provide evidence that purified reverse transcriptase alone is enzymatically sufficient for correct RNA-directed DNA synthesis. Successful achievement of these goals will help to demonstrate the usefulness and validity of the reconstructed reaction as a tool in detailed analysis of retrovirus reverse transcription.
Chapter II

MATERIALS AND METHODS

MATERIALS

Unless otherwise specified, all general chemicals were of the highest purity available. The water used for preparation of reagents was glass distilled from distilled, deionized tap water. All glassware, teflon coated stir bars, and metal spatulas were baked at 225 °C for a minimum of five hours. Reagents were further purified whenever possible by filtration through 0.45 micron nitrocellulose membrane filters and/or through 0.25 mm diameter glass fiber filters.

The pH of all buffers was measured at 20 °C unless otherwise noted. Guanidine hydrochloride was obtained from Heico, Inc. TEMED (N,N,N',N'-tetramethylethylenediamine) and 98% formamide was purchased from the Aldrich Chemical Company. The formamide was deionized prior to use by mixing one gram of mixed bed ion exchange resin (AG501-X8, 20-50 mesh) per 10 milliliters formamide for 30 minutes. The resin was then replaced by fresh AG501-X8 resin and the slurry mixed again for 30 minutes. The deionized formamide was then filtered through two glass fiber filters and stored at -20 °C. AG501-X8, 20-50 mesh, mixed bed ion exchange resin, acrylamide, and methylene-bis-acrylamide were bought from Bio-Rad Laboratories. 3 MM chromatography paper and DE81 cellulose filters were purchased from Whatman Chemical Separation, Ltd. Glass fiber filters (0.25 mm dia.) were obtained from Enzo Biochem, Inc. Nitrocellulose filters (BA85, 0.45 micron) and S&S 589 WR paper were purchased from
Schleicher and Schuell, Inc. Sodium dodecyl sulfate (SDS) was obtained from Mallinckrodt. Type I agarose (low EEO), type VII agarose (low gelling temperature), dithiothreitol (DTT), dimethylsulfoxide (DMSO), bromophenol blue, calf thymus DNA (type I), PIPES (piperazine-N,N'-bis 2-ethane sulfonic acid), Tris (Trizma base; Tris (hydroxymethylaminomethane), 2,5-diphenyloxazole (PPO), dextran sulfate (M_r=500,000), bovine serum albumin (fraction V), salicylic acid, ficoll (type 400), yeast RNA (type X), diethyl pyrocarbonate, and polyvinyl pyrrolidine (PVP 400) were all obtained from Sigma Chemical Company. Aquasol II, α-[32P]-deoxyribonucleoside 5'-triphosphates (600-800 Ci/mmol), and γ-[32P]-adenosine 5'-triphosphate (2900 Ci/mmol) were purchased from New England Nuclear. [³H]-deoxyribonucleoside 5'-triphosphates were bought from ICN Chemical and Radioisotope Division. Oligothymidylic acid (oligo(dT) _{12-18} ) and polyadenylic acid (poly(rA)) were purchased from Collaborative Research, Inc. Unlabeled deoxyribonucleoside 5'-triphosphates, polynucleotide kinase (from T₄-infected Escherichia coli, minimal nuclease), and bacterial alkaline phosphatase (Escherichia coli, minimal nuclease) were obtained from P-L Biochemicals. Urea (ultra-pure grade) was purchased from Schwartz-Mann. Hind III-digested λ phage DNA markers, Hae III-digested φX-174 phage DNA markers, and all restriction endonucleases were obtained from New England Biolabs, Inc. G-50 Sephadex was purchased from Pharmacia Fine Chemicals. Lanthanum acetate was obtained from Tridon Chemical Company. Xylene cyanole FF, 8-hydroxyquinoline, and XR x-ray film was purchased from the Eastman Kodak Chemical Company. Sucrose (RNase free), density gradient centrifugation grade, was supplied by Beckman Instruments, Inc. NCS tissue solubilizer was bought from the Amersham Corp. Phenol was
purchased from the J. T. Baker Chemical Company. The phenol was further purified by distillation (bp 175 °C). 0.1% (w/v) 8-hydroxyquinoline was added as a preservative and the phenol was stored at -20 °C.

METHODS

Purification of AMV Reverse Transcriptase

Purification of AMV (BAI strain A) was performed essentially as described previously (Rosok and Watson, 1979; Olsen, 1982) from frozen plasma of leukemic chicks obtained from the Office of Program Resources and Logistics, Virus Cancer Program, National Cancer Institute, Bethesda, Maryland. The activity of reverse transcriptase was monitored prior to and following each purification step by the rA-dT assay procedure described later in Methods. After the final purification step was completed, active fractions were stored at -20 °C in a buffer containing 50% (v/v) glycerol, 150 mM potassium phosphate (pH 8.0), and 10 mM DTT.

Nucleic Acid Purification

SDS-Phenol Extraction

To extract proteins from solutions containing nucleic acids, a modification of the phenol extraction procedure of Brawerman (1974) was employed. Enzymatic reactions were terminated by the addition of sufficient EDTA and SDS to achieve final concentrations of 20 mM and 1% (w/v), respectively. One volume of redistilled phenol containing 0.1% (w/v) quinoline that had been pre-equilibrated in extraction buffer (10 mM Tris-HCl (pH 8.8), 100 mM NaCl, and 5 mM EDTA) was added and the mixture vortexed briefly. The mixture was centrifuged at room temperature in a table top centrifuge for one minute, and the upper aqueous phase removed. One volume of fresh extraction buffer was added to the phenol phase, and
the extraction step repeated. The second aqueous extract was combined with the first extract. When recovery of nucleic acid was paramount to further studies, the phenol phase was extracted a third time with one volume of fresh extraction buffer. The aqueous extracts could be purified further by G-50 Sephadex chromatography or precipitated by the addition of 0.1 volume 4 M NaCl and 3 volumes absolute ethanol at -20 °C for a minimum of three hours. Alternatively, the time required for precipitation could be decreased to 30 minutes by storage at -70 °C. Recovery of the precipitate was accomplished by centrifugation at 20,000 x g for 45 minutes at 2 °C. Trace amounts of phenol remaining with the pellet could be removed by washing the pellet with cold 70% (v/v) ethanol and 40 mM NaCl, followed by recentrifugation at 2 °C for 20 minutes at 20,000 x g. The pellet was then decanted, aspirated to dryness, and finally redissolved in the desired buffer.

Oligo(dT)-Cellulose Chromatography

Separation of viral RNA into poly(A)-deficient (poly(A)⁻) and poly(A)-containing (poly(A)⁺) pools was performed by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972; Bantle et al., 1976). Viral RNA that had been precipitated in the presence of 70% (v/v) ethanol and 0.4 M NaCl was recovered by centrifugation as described previously, and the pellet dissolved in 20 mM Tris-Cl (pH 7.4), 10 mM EDTA, and 0.2% (w/v) SDS. The dissolved viral RNA was heated at 60 °C for five minutes followed by quick cooling on ice. Enough 4 M NaCl was added to adjust the final concentration of NaCl to 0.5 M. The concentration of viral RNA was determined by measuring the absorbance at 260 nm (extinction coefficient 20 mg⁻¹), and the entire mixture applied to an oligo(dT)-cellulose column
(0.9 x 1.5 cm) pre-equilibrated in buffer A (10 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 5 mM EDTA, and 0.2% (w/v) SDS). After washing the oligo(dT)-cellulose column with 10 column volumes of buffer A to remove poly(A)$^-\text{viral RNA}$, the poly(A)$^+\text{viral RNA}$ was eluted by the addition of 5-10 column volumes of buffer C (10 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.2% (w/v) SDS) at a flow rate of 0.5-1.0 ml/minute. Finally, the oligo(dT)-cellulose column was washed with 5 column volumes of double-distilled H$_2$O (dd H$_2$O). Fractions containing poly(A)$^+\text{RNA}$ were determined by measuring the absorbance at 260 nm, and pooled. The oligo(dT)-cellulose column was re-equilibrated in buffer A, and the pooled poly(A)$^+\text{viral RNA}$ re-applied to the column after adjusting the NaCl concentration to 0.5 M. The oligo(dT)-cellulose column was washed again with buffer A as described previously, and the poly(A)$^+\text{viral RNA}$ eluted by the addition of buffer C. Fractions containing poly(A)$^+\text{viral RNA}$ were pooled, precipitated by the addition of 0.1 volume 4 M NaCl and 3 volumes absolute ethanol, and stored at -20 °C.

**Purification of the AMV 35S RNA-tRNA$^{Trp}$ Subunit**

AMV 35S RNA containing the tRNA$^{Trp}$ primer was purified from either freshly detergent-disrupted virions or from frozen FNA-containing pellets remaining after the detergent disruption of virions exactly as described by Olsen (1982).

Briefly, 60-70S RNA was purified by velocity sedimentation centrifugation in 10-30% (v/v) glycerol gradients containing 10 mM Tris-HCl (pH 7.4), 40 mM LiCl, 5 mM EDTA, and 0.2% (w/v) SDS, on an SW27 rotor at 26,000 rpm for 3.75 hr at 21 °C. The fractions containing the 60-70S RNA were pooled, and fractionated into poly(A)$^-\text{and poly(A)}^+\text{pools by}$
oligo(dT)-cellulose chromatography. The poly(A)$^+$ 60-70S RNA was heated gently to dissociate the subunits, and fractionated twice in 10-30% (v/v) glycerol gradients at 37,000 rpm for 5.5 hr at 21 °C in a SW41 rotor. The 35S RNA-containing fractions were pooled, and stored at -20 °C in 70% (v/v) ethanol and 0.4 M NaCl.

Reverse Transcriptase Reactions

Oligo(dT)-poly(rA)

To determine the relative activity of reverse transcriptase fractions and as a check on enzyme activity after long term storage, the following assay conditions were used: various amounts of purified reverse transcriptase were incubated for 10 minutes at 37 °C in a 50 μl reaction mixture which contained 50 mM Tris-HCl (pH 8.1), 8 mM MgCl$_2$, 10 mM DTT, 40 mM NaCl, 0.2 mM dATP, 0.05 mM $[^3]$H-dTTP (60-120 cpm/pmol), and 0.6 μg/ml poly(rA)-oligo(dT)$_{12-18}$ template-primer. The poly(rA)-oligo(dT)$_{12-18}$ was prepared by mixing poly(rA) with oligo(dT)$_{12-18}$ to final concentrations of 125 μg/ml and 25 μg/ml, respectively, in 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 1 mM EDTA. The mixture was heated briefly for 1.5 minutes at 97 °C, followed by incubation for 30 minutes at 37 °C to allow annealing of the template-primer. Reactions were terminated by pipetting the entire reaction mixture on DE81 filter paper. Unincorporated $[^3]$H-dTTP was removed by washing the filters six times in 5% (w/v) Na$_2$HPO$_4$ for 4 minutes per wash (Blatti et al., 1970). The filters were then dried and placed in three ml of a toluene-based liquid scintillation cocktail containing 0.3% (w/v) PPO, 0.008% (w/v) bis-MSB, 2.5% (v/v) NCS tissue solubilizer, and 0.035% (v/v) dd H$_2$O. The extent of $[^3]$H-dTTP incorporated into polydeoxyribonucleotide (poly DNA) was
measured by liquid scintillation spectrophotometry using a Beckman LS-230 liquid scintillation counter.

Using these conditions, one unit of DNA polymerase activity is defined as the amount of enzyme required to catalyze the incorporation of one pmol of dTTP per minute into polymeric DNA.

\[ 35S \text{RNA-}tRNA^{Trp} \text{ Template-Primer} \]

Synthesis of genome-length complementary DNA (cDNA) copies of the 35S RNA template was performed using the following reaction conditions: 50 mM potassium phosphate (pH 8.0); 58 mM NaCl; 5 mM MgCl\(_2\); 10 mM DTT; 20 µg/ml 35S RNA-tRNA\(^{Trp}\); 0.03 mM \(\alpha\)^-[\(^{32}\)P]-dCTP (1500-2000 cpm/pmol); 0.2 mM each of dATP, dGTP, and dTTP; and saturating amounts of purified reverse transcriptase. The enzyme storage buffer contributed 30 mM potassium phosphate (pH 8.0) to the reaction mixture, and is included in the total phosphate concentration of 50 mM. When \(^3H\)-dCTP was substituted for \(\alpha\)^-[\(^{32}\)P]-dCTP, the specific activity was increased to 3000-3300 cpm/pmol. The reaction was incubated for 2 hours at 39 °C, and terminated by the addition of EDTA and SDS to 20 mM and 0.1% (w/v), respectively. The mixture was then phenol extracted 2-3 times as described previously.

When necessary, the DNA products from the reconstructed reaction were purified further by gel exclusion chromatography on a G-50 Sephadex column (1.5 x 24 cm) in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, and 0.1% (w/v) SDS. One ml fractions were collected and a small aliquot of each fraction spotted on DE81 (or 3 MM) filter paper, dried, and counted in a liquid scintillation counter. Fractions containing radio-labeled DNA were pooled, concentrated with 2-butanol (Stafford and Bieber,
1975), and precipitated with ethanol. When the DNA products from the reconstructed reaction were to be used in further experiments requiring the absence of viral RNA, the RNA was hydrolyzed overnight at room temperature by the addition of NaOH to a final concentration of 0.3 N immediately prior to the ethanol precipitation step. Following hydrolysis, the mixture was neutralized, and precipitated with ethanol. Neutralization was accomplished by adding 0.3 N HCl and sufficient 1 M Tris-HCl (pH 7.4) to achieve a final pH value of approximately 7.0.

When the buffer conditions were modified to contain 50 mM Tris-HCl (pH 8.1), the concentration of phosphate was reduced to 30 mM (at pH 8.0), and the NaCl concentration was increased to 92 mM. Otherwise the reaction conditions were identical to the phosphate-buffered system.

**35S RNA·oligo(dT) Template-Primer**

Synthesis of cDNA using 35S RNA template containing the poly(A) tail and oligo(dT)12-18 as primer was accomplished in identical reaction mixtures as used when tRNATrp was the primer, except that the 35S RNA concentration was 25 µg/ml and the concentration of oligo(dT)12-18 was 5-10 µg/ml. The reaction was incubated at 37 °C for 60-90 minutes. All other steps were identical to those described for the tRNATrp-primed reaction.

**cDNA as Template**

Synthesis of the second strand of DNA was necessary to provide a suitable double-stranded DNA substrate for restriction mapping. Synthesis of the second strand (plus strand) was accomplished as follows: 1-10 ng of cDNA that had been pre-sized by alkaline sucrose velocity sedimentation centrifugation in a SW60 rotor, was resuspended in a 20 µl
reaction mixture containing 10 mM Tris-HCl (pH 8.1), and 5-10 μg of S1 nuclease-digested calf thymus DNA primers. The mixture was heated for 3 minutes at 97 °C, and quick cooled on ice. The volume was increased to 40 μl by the addition of 10 μl of 5X basal mixture (250 mM Tris-HCl (pH 8.1), 200 mM NaCl, 40 mM MgCl₂, and 50 mM DTT), and 10 μl of 5X 4 dNTP mix (0.15 mM [³H]-dCTP, and 1 mM each of dATP, dGTP and dTTP). The mixture was heated for 30 min at 39 °C to allow annealing of the calf thymus DNA primers to the cDNA template. Ten μl of reverse transcriptase-containing enzyme buffer was added, and the reaction incubated for 90 min at 39 °C. The reaction was terminated by the addition of EDTA and SDS to 20 mM and 0.1% (w/v) respectively, and extracted 2-3 times with phenol. When the DNA products were to be utilized for restriction mapping, 3-5 μg of heat denatured calf thymus DNA was added to the phenol extracts to act as a carrier. The DNA products were further purified by G-50 Sephadex chromatography, and precipitated with ethanol as described previously.

**Optimization of the Reverse Transcriptase Assay**

Titration of reverse transcriptase established the relative activity of each pool of enzyme as well as the concentration of enzyme required to achieve saturation of the template. Use of the poly(rA)-oligo(dT) assay allowed the initial activity of the reverse transcriptase pools to be determined (Fig. 5a). The value obtained, 5.8-6.0 units of DNA polymerase activity per μl of enzyme buffer, was typical of the pools of reverse transcriptase utilized. Use of the 35S RNA-oligo(dT)₁₂-₁₈ template-primer system resulted in a ten fold decrease in DNA synthesis (Fig. 5b). At saturating levels of reverse transcriptase, the value of 0.6 units of DNA polymerase activity was somewhat greater than the 0.3
Fig. 5. Titration of Reverse Transcriptase With Different Template·Primers. Various amounts of purified reverse transcriptase was incubated with different template·primers in standard 50 µl reaction mixtures as described in Methods. Total amount of DNA synthesized was determined by the DE81 filter binding assay.

A. Poly(rA)·oligo(dT) as template·primer. Specific activity of $[^3H]$-dTTP was 105 cpm/pmol. Reaction mixtures were incubated for 10 minutes at 37 °C.

B. 35S RNA·oligo(dT) as template·primer. Specific activity of $[^3H]$-dCTP was 200 cpm/pmol. Reaction mixtures were incubated at 39 °C for 60 minutes. Reactions were buffered in 50 mM Tris-HCl (pH 8.1).

C. 35S RNA·tRNA$^\text{Irr}$ as template·primer. Specific activity of $[^3H]$-dCTP was 3000 cpm/pmol. Reactions were incubated in 50 mM potassium phosphate buffer (pH 8.0) for 120 minutes at 39 °C.

- 4 mM Na$_4$P$_2$O$_7$
- 0 mM Na$_4$P$_2$O$_7$
units of DNA polymerase activity obtained with the potassium phosphate-
buffered, tRNA\textsuperscript{Trp}-primed reconstructed reaction (Fig. 5c). Similar
values have been found previously (Olsen, 1982).

In all further experiments utilizing reverse transcriptase, concentrations of enzyme which resulted in saturation of the RNA template
(the plateau region in Fig. 5c) were used in order to maintain consistent results.

**Sizing of Nucleic Acids**

**Alkaline Sucrose Velocity Sedimentation Centrifugation**

DNA products from the reconstructed reaction that had been stored at
-20 °C in 70% (v/v) ethanol and 0.4 M NaCl were recovered by centrifuga-
tion at 20,000 x g for 45 minutes at 2 °C, aspirated dry, and resus-
pended in 0.1 ml of 0.3 N NaOH and 4 mM EDTA. The sample was layered on
4.4 ml 5-20% (w/v) sucrose gradients containing 0.69 M LiCl, 0.3 N KOH,
and 5 mM EDTA. The gradients were centrifuged for 4.5 hours at 54,000
rpm in a SW60 rotor at 21 °C. \(^{32}\text{P}\)-labelled \(\lambda\) phage DNA markers were
run on a parallel gradient. Fractions (180 \(\mu\)l) were collected from the
bottom of each gradient by needle puncture, and a small aliquot of each
fraction was pipetted on 3 M M filter paper, dried, and counted in a liquid
scintillation counter. DNA-containing fractions were diluted by the
addition of 4 volumes of 10 mM Tris-HCl (pH 8.1), neutralized, and pre-
cipitated with ethanol as described. When sizing of the DNA products
were preparatory to synthesis of the plus strand of DNA, 5-10 \(\mu\)g of calf
thymus DNA primers were added immediately prior to the ethanol precipi-
tation step. The fractions were stored at -20 °C until required.
Alkaline Agarose Gel Electrophoresis

Sizing of DNA was performed using horizontal alkaline agarose gel electrophoresis (McDonnell et al., 1975) or by vertical tube alkaline agarose gel electrophoresis. DNA ranging in size from 0.5-10 kb were routinely sized on 200 ml horizontal 1.2% (w/v) agarose gels containing 30 mM NaOH and 2 mM EDTA (E buffer). Gels were prepared by dissolving 2.4 g agarose (low electroendoosmosis) in 173 ml dd H₂O by heating the solution to boiling. The dissolved agarose was cooled to 45 °C, and 20 ml of warm 0.3 N NaOH and 20 mM EDTA added. The agarose solution was mixed carefully to avoid air bubbles, and poured onto a 20 x 28 cm plexiglass casting tray. After standing for one hour at room temperature, the agarose had solidified sufficiently to allow handling. The gel was submerged in electrophoresis buffer, and the samples loaded. Radiolabeled samples were dissolved in 10-15 μl sample buffer (10% (v/v) glycerol, 30 mM NaOH, 4 mM EDTA, 0.025% (w/v) bromophenol blue, and 0.025% (w/v) xylene cyanole FF) and heated at 37 °C for 10 minutes immediately prior to loading. The samples were electrophoresed at 50 volts (constant voltage) for 18-22 hours. The gel was then rinsed with dd H₂O to remove excess buffer, and dried on Gel-Bond over gentle heating according to the manufacturers instructions. When dry, the gel was ready for autoradiography.

When detailed resolution was not required, electrophoresis was also performed using a 20 ml alkaline 1.2% (w/v) agarose "mini-gel" system. The mini-gel was prepared exactly as described above for the 200 ml gel. Samples were dissolved in 5-8 μl sample buffer. Running conditions were modified by decreasing the time for electrophoresis to 90-120 minutes.
Otherwise all other operations were the same as for the 200 ml gel.

Alternatively, vertical tube alkaline agarose gels were used to obtain a more quantitative indication of DNA sizes. Alkaline 0.7% (w/v) agarose gels were cast in 11.0 x 0.5 cm (I.D.) glass tubes that had the bottoms sealed with dialysis tubing. The samples were overlaid on top of each gel and fractionated for 5.75 hours at 25 volts (constant voltage). The gel was sliced into 1.5 mm thick slices, and each slice dissolved in 3 ml of Aquasol II. The amount of radiolabeled DNA in each slice was determined using a liquid scintillation counter.

**4.8% (w/v) Polyacrylamide-7 M Urea Gel Electrophoresis**

DNA species less than 1 kilobase (kb) in length were analyzed using denaturing polyacrylamide gels containing 7 M urea (Maniatis et al., 1976). Twenty-five μl of a solution containing 7 M urea, 0.12% (w/v) bis acrylamide, 89 mM Tris-borate (pH 7.6), 89 mM boric acid, 10 mM EDTA, and 0.064% (w/v) fresh ammonium persulfate, was stirred until dissolved. After addition of 15 μl of TEMED, the gel was cast, and allowed to polymerize for 1-2 hours at room temperature. The radiolabeled nucleic acids were dissolved in 30-40 μl of sample buffer (95% (v/v) deionized formamide, 0.01% (w/v) bromophenol blue, and 0.1% (w/v) xylene cyanole FF), heated for 3 minutes at 97 °C, and quick cooled on ice. The samples were loaded and fractionated for 2.75 hours at 12 mA (constant current). The gel was then fixed for 30 minutes in a solution containing 1% (w/v) lanthanum acetate and 1% (v/v) acetic acid, dried on 3 MM paper under a vacuum, and subjected to autoradiography.

**Fluorography**

Alkaline agarose gels containing [³H]-labeled DNA were pretreated
prior to drying by fluorography. The prewashed gel was fixed in 80 ml of 1% (w/v) lanthanum acetate and 1% (v/v) glacial acetic acid for 20 minutes. The lanthanum acetate/acetic acid solution was drained, and the gel neutralized by adding 320 ml of 1.56% (w/v) ammonium acetate. After soaking for 20 minutes, the ammonium acetate solution was removed and the gel immersed in 320 ml of 15% (w/v) salicylic acid for 30 minutes. Finally, the gel was washed for 5 minutes in 200 ml of a solution containing 5% (v/v) glacial acetic acid. The gel was then sandwiched between a sheet of 3 MM paper and saran wrap, and dried under reduced pressure.

**Autoradiography**

Detection of radiolabeled products was accomplished by autoradiography. Dried gels containing $^{32}$P-labeled nucleic acids were exposed to Kodak XR film using a film cassette with an image intensifying screen at -70 °C. The film was developed and processed according to the manufacturers instructions under a Kodak safety lamp. The developed film was washed in cold running water, air dried, and mounted. When $^3$H was the radiolabel, the process was modified by presensitizing the film prior to exposure. The film was exposed to a brief (less than 1 msec) electronic flash, followed by exposure to the dried gel. All other steps were the same as for $^{32}$P-labeled products.

**RNA•DNA Hybridization**

To determine the extent of plus DNA synthesized in the phosphate-buffered reconstructed reaction, the DNA products were hybridized with at least a 50-fold mass excess of viral 35S RNA. Twenty-five μl of a solution containing purified DNA, 35S RNA, and 10 mM potassium phosphate
(pH 6.1) was heated for 3 minutes at 97 °C, and quick cooled on ice. An equal volume of 2X hybridization buffer (10 mM potassium phosphate (pH 6.1), 1 M NaCl, and 2 mM EDTA) was added, and mixed thoroughly. The mixture was incubated at 69 °C \( (C_r o t = 0.45 \text{ mol·sec·l}^{-1}) \). Three volumes of absolute ethanol were added and the mixture stored at -20 °C. The extent of plus DNA was determined by resistance to S1 nuclease digestion.

Alternatively, when equilibrium density centrifugation was used for separation of plus DNA from cDNA, the purified DNA products from the reconstructed reaction were dissolved in 0.2 ml of a solution containing 80% (v/v) deionized formamide, 40 mM PIPES-NaOH (pH 6.4), 1 mM EDTA, and a 30-fold mass excess of viral 35S RNA. The solution was briefly heated at 85 °C for 3 minutes followed by quick cooling on ice. Sufficient 4 M NaCl was added to achieve a final NaCl concentration of 0.4 M, and the nucleic acids allowed to hybridize at 52 °C \( (C_r o t = 0.18 \text{ mol·sec·l}^{-1}) \). Under these conditions RNA-DNA hybrids are allowed to form but DNA-DNA duplexes do not (Casey and Davidson, 1977). The mixture was then cooled on ice. An equilibrium density gradient was prepared using a modification of the method of Enea and Zinder (1975); 8.6 g of CsCl and 1.6 g of guanidinium hydrochloride dissolved in 10 ml of 10 mM Tris-HCl (pH 7.4). The previously cooled hybridization mixture was then added to 4.7 ml of the equilibrium density gradient, and centrifuged in a polyallomer tube in a SW60 rotor for 48 hr at 21 °C. Under these conditions, RNA-DNA hybrids pellet at the bottom of the tube and unhybridized single-stranded DNA is found approximately two-thirds down the gradient. Fractions were collected from the bottom by needle puncture, and the fractions containing unhybridized single-stranded DNA were pooled, desalted on a G-50 Sephadex
column, and finally precipitated with ethanol. The size of the unhybridized DNA was determined by alkaline 0.7% (w/v) agarose gel electrophoresis as described previously.

**SI Nuclease Digestion**

Standard reaction mixtures consisted of 0.1-1.0 ml of a solution containing 0.2 M sodium acetate (pH 4.4), 5 mM ZnSO₄, 5 µg/ml native calf thymus DNA, radiolabeled DNA, and predetermined amounts of SI nuclease. When SI nuclease digestion was used to determine the extent of RNA-DNA hybridization, the reaction also included 0.5-2.0 µg of AMV 35S RNA. Digestion was carried out at 42 °C for 75-90 minutes. The amount of DNA resistant to SI nuclease digestion was determined by collecting the samples on nitrocellulose filters, washing extensively with cold 5% (w/v) trichloroacetic acid, and counting the insoluble nucleic acid in a liquid scintillation counter.

**5'-End Labeling**

Hind III-digested λ phage and Hae III-digested φX-174 phage DNA markers were employed as molecular weight standards for use in centrifugation and electrophoresis. The DNA markers were labeled with [³²P] at their 5'-ends in a two step reaction by a modification of the method of Chaconas and Van de Sande (1975).

A predetermined amount of DNA markers were precipitated in the presence of 70% (v/v) ethanol and 0.4 M NaCl at -20 °C, and recovered by centrifugation at 20,000 x g for 30 minutes at 2 °C. The DNA-containing pellet was resuspended in 43 µl of 10 mM Tris-HCl (pH 8.1), and 2 µl (4-5 units) of *Escherichia coli* bacterial alkaline phosphatase added. The reaction was incubated for 30 minutes at 37 °C. Five µl of 60 mM
potassium phosphate (pH 7.2) was added to inhibit the bacterial alkaline phosphatase, and the DNA directly reacted with γ-[\(^{32}\)P]-ATP and polynucleotide kinase. End labeling of dephosphorylated DNA was accomplished in 100 μl reactions containing 0.5 mM spermidine, 6 mM potassium phosphate (pH 7.2), 50 mM Tris-HCl (pH 8.8), 5 mM MgCl\(_2\), 10 mM DTT, sufficient γ-[\(^{32}\)P]-ATP (greater than 2500 Ci/mmol) to achieve a ratio of 5 pmol γ-[\(^{32}\)P]-ATP/pmol 5'-OH ends, and 4-5 units of polynucleotide kinase. The reaction was incubated for 35 minutes at 37 °C, terminated by the addition of EDTA and SDS to 20 mM and 1% (w/v), respectively, and then extracted with phenol. Unincorporated γ-[\(^{32}\)P]-ATP was separated from the 5'-end labeled DNA by gel filtration using G-50 Sephadex. The 5'-end labeled DNA in the excluded volume was pooled, and concentrated with 2-butanol to a final volume of 0.2-0.5 ml. One volume of absolute ethanol was added, and the 5'-end labeled DNA stored at -20 °C.

Restriction Endonuclease Mapping

Restriction endonuclease mapping of the [\(^{32}\)P]-labeled cDNA transcripts was performed by digestion of cDNA-plus DNA duplexes using the buffer conditions recommended by the supplier with the exception that bovine serum albumin was not used. Standard reactions consisted of 50 μl of a mixture containing 1-10 ng cDNA-plus DNA duplex, the recommended buffer conditions, 100 μg/ml calf thymus DNA, and 2-4 fold excess of the appropriate restriction endonuclease. The reactions were incubated overnight at 37 °C, terminated by the addition of EDTA and SDS to 20 mM and 1% (w/v) respectively, and extracted with phenol. The phenol extracts were precipitated at -20 °C by the addition of 0.1 volume 4 M NaCl, 50-100 μg of purified yeast RNA, and 3 volumes of absolute ethanol.
Sizing of the restriction endonuclease digested duplexes was done on alkaline 1.2% (w/v) agarose gels followed by autoradiography as described previously.

Mapping of the restriction sites detected on the cDNA transcripts was done by selection and isolation of different size classes of cDNA by centrifugation on alkaline sucrose velocity gradients prior to second-strand DNA synthesis. Orientation of the map was accomplished by taking advantage of the fact that all cDNA synthesized in the reconstructed reaction, regardless of size, is homogenous with respect to its 5'-end sequences. Any heterogeneity is the result of differing sizes corresponding to the 3'-ends of the cDNA transcripts. Therefore, any restriction fragment should be easily mapped if the sizes of the original cDNA transcripts are judiciously selected. For example, of restriction endonuclease digestion of a 1.0 kb cDNA specie yields two fragments of lengths 0.4 and 0.6 kb, the map may not be able to be orientated properly. However, if digestion of a 2.0 kb specie of cDNA with the same restriction endonuclease yields two fragments of lengths 0.6 and 1.4 kb, then the cDNA template contains a restriction endonuclease site for that enzyme 0.6 kb from the 5'-end of the cDNA.

Preparation of Nucleic Acid Probes

cDNA_3.

Synthesis and purification of [^{32}P]-cDNA complementary to the 3'-end of AMV 35S RNA was done essentially as described by Tal et al., (1975). 50 μg of viral 8-10S poly(A)⁺ RNA was incubated at 37 °C for 60 minutes in a 0.5 ml reaction volume containing: 50 mM Tris-HCl (pH 8.1); 40 mM NaCl; 10 mM MgCl₂; 10 mM DTT; 50 μg/ml actinomycin D; 4 μg/ml
oligo(dT)_{12-18}; 1 \mu M \alpha-[^{32}P]-dCTP (600 Ci/mmol); 1 mM each of dATP, dGTP, and dTTP; and saturating amounts of purified reverse transcriptase. The reaction was terminated by the addition of EDTA and SDS to 20 mM and 1% (w/v), respectively. The entire reaction was then heated at 97 °C for 5 minutes, and quick cooled on ice.

Purification of the cDNA_3, probe was done in a two step procedure. Sixty-five \mu g of poly(rA) was added to the chilled mixture, and sufficient 4 M NaCl was added to adjust the final concentration of NaCl to 0.5 M. The mixture was incubated at 47 °C for five minutes, followed by 45 minutes at room temperature. The sample was passed twice through an oligo(dT)-cellulose column as described previously. The DNA that bound to the oligo(dT)-cellulose column via the poly(rA) bridge was pooled, and alkali-hydrolyzed overnight at room temperature. The solution containing the cDNA_3, probe was neutralized, and desalted on a G-50 Sephadex column. Fractions containing the cDNA_3, probe were pooled, reduced in volume, and finally stored at -20 °C in 50% (v/v) ethanol. When sized by alkaline agarose gel electrophoresis, the cDNA_3, probe had a length of 275-325 nucleotides.

cDNA_{rep}

[^{32}P]-cDNA_{rep}, a cDNA probe representative of the entire AMV genome, was prepared by a modification of the method of Taylor et al., (1976) as follows: 10 \mu g of AMV 35S RNA was incubated for 2 hours at 37 °C in a 100 \mu l reaction mixture which contained: 100 \mu g/ml actinomycin D; 1 mg/ml calf thymus DNA primers; 50 mM Tris-HCl (pH 8.1); 10 mM DTT; 8 mM MgCl_2; 0.4 mM \alpha-[^{32}P]-dCTP (specific activity = 36,000 cpm/pmol); 0.25 mM each of dATP, dGTP, and dTTP; 40 mM NaCl; and saturating amounts of purified
reverse transcriptase. The reaction was terminated by the addition of sufficient EDTA and SDS to achieve a final concentration of 20 mM and 1% (w/v), respectively. The mixture was phenol extracted, and hydrolyzed overnight in 0.3 N NaOH. Following neutralization, the mixture was purified by G-50 Sephadex gel exclusion chromatography. Fractions containing the $[^{32}P]$-labeled cDNA<sub>rep</sub> probe were pooled, and stored at -20 °C in 50% (v/v) ethanol.

**8-10S RNA**

Detection of cDNA sequences localized at the LTR was accomplished by employing 5'-$[^{32}P]$-8-10S poly(A)<sup>+</sup> RNA as a probe. 8-10S RNA obtained from the purification procedure of 35S RNA-tRNA<sub>Trp</sub> was pelleted, and resuspended in 200 μl of RNA buffer (10 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% (w/v) SDS). The 8-10S viral RNA was centrifuged for 21 hours at 30,000 rpm in 12 ml 10-30% (v/v) glycerol gradients (10 mM Tris-HCl (pH 7.4) 5 mM EDTA, 40 mM LiCl, and 0.1% (w/v) SDS) in a SW41 rotor at 21 °C. One-half ml fractions were collected from the bottom of the gradient by needle puncture. Fractions containing viral RNA from the back slope of the 8-10S RNA peak were pooled, precipitated with ethanol, and stored overnight at -20 °C. Following centrifugation to recover the sub 8-10S RNA, the RNA-containing pellet was dissolved in one ml of 20 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.2% (w/v) SDS, and chromatographed on an oligo(dT)-cellulose column as described previously. Fractions containing poly(A)<sup>+</sup> RNA were pooled, precipitated with ethanol, and stored at -20 °C until ready for 5'-end labeling.

The sub 8-10S poly(A)<sup>+</sup> viral RNA was 5'-end labeled following a modification of the method used to prepare phage marker DNA. Approxi-
mately 5 μg of sub 8-10S poly(A)$^+$ RNA was incubated for one hour at 37 °C in a 30 μl reaction mixture which contained: 10 mM Tris-HCl (pH 8.1), 0.1% (w/v) SDS, and 5-10 units of bacterial alkaline phosphatase. The reaction was terminated by the addition of EDTA and SDS to 20 mM and 1% (w/v), respectively, and extracted with freshly equilibrated phenol. The dephosphorylated sub 8-10S poly(A)$^+$ RNA was precipitated at -70 °C for 45 minutes by the addition of 0.1 volume 4 M NaCl and 3 volumes of absolute ethanol. The RNA was recovered by centrifugation at 20,000 x g for 45 minutes at 2 °C, dried by aspiration, and resuspended in 80 μl of a mixture containing: 50 mM Tris-HCl (pH 8.8); 5 mM potassium phosphate (pH 8.0), 0.5 mM spermidine; 10 mM DTT; 100 μCi γ-$^{32}$P-ATP (2700 Ci/mmol); 10 mM MgCl$_2$; and 4 units of T4 bacteriophage polynucleotide kinase. The reaction was incubated for 35 minutes at 37 °C, terminated by the addition of EDTA and SDS to 20 mM and 1% (w/v), respectively, and extracted with phenol. The 5'-$^{32}$P-labeled RNA probe was purified by G-50 Sephadex chromatography, and stored at -20 °C in 50% (v/v) ethanol. As a final purification step, the $^{32}$P-labeled sub 8-10S poly(A)$^+$ probe was recentrifuged on a 4.4 ml 10-30% glycerol gradient (10 mM Tris-HCl (pH 7.4), 40 mM LiCl; 5 mM EDTA, and 0.2% (w/v) SDS) at 54,000 rpm for 24 hours at 21 °C in a SW60 rotor. One hundred and fifty μl fractions were collected from the bottom of the gradient by needle puncture, and the 5'-$^{32}$P-labeled RNA from the front slope of the sub 8-10S RNA peak were pooled, and stored at -20 °C in 50% (v/v) ethanol.

**Southern Blotting**

*NBMPAPER PREPARATION*

Nitrobenzyloxyethyl (NBMP)-paper was prepared essentially as
described previously (Alwine et al., 1977; Wahl et al., 1979; Olsen; 1982). Sheets of Schleicher and Schuell 589 paper (8 x 12 cm) were thoroughly soaked in a solution containing 0.94 mg/cm² sodium acetate, 3 mg/cm² N-(3-nitrobenzyloxymethyl)-pyridinium chloride, and 0.037 ml/cm² dd H₂O. The soaked paper was placed on glass plates, and any air bubbles under the paper removed. The paper was dried at 60 °C, and then baked for 35-40 minutes at 130 °C. The paper was washed twice for 20 minutes per wash in dd H₂O, followed by washing twice for 20 minutes per wash in acetone. The NBM-paper was air-dried, and stored in a dessicator at 4 °C until used.

**DBM-Paper Preparation**

Immediately prior to reaction with the fractionated single-stranded nucleic acids, the NBM-paper was reduced to aminobenzyloxymethyl (ABM)-paper. The NBM-paper was soaked in a solution containing 100 ml freshly prepared 20% (w/v) sodium hydrosulfite at 60 °C for 30 minutes. The ABM-paper was washed 5 times rapidly with dd H₂O, and once for 3 minutes at room temperature with 100 ml of 3% (v/v) acetic acid. All subsequent steps were performed at 4 °C using prechilled solutions. The ABM-paper was washed 5 times rapidly with dd H₂O, and converted to the DBM form by soaking for 30 minutes at 4 °C in 102.6 ml of a freshly prepared solution containing 1.2 N HCl and 26 mg of NaNO₂. The DBM-paper was washed rapidly 4 times with dd H₂O (100 ml/wash) and twice for 5 minutes per wash with 100 ml of 1 M sodium acetate (pH 4.0). At this point the DBM-paper is slightly yellow and was reacted with the gel within two minutes.
Transfer of DNA to DBM-Paper

The sample DNA to be transferred to the DBM-paper was fractionated on a horizontal alkaline agarose gel as described previously. The end of electrophoresis was timed so that the gel was ready for transfer at the same time the DBM-paper was ready. Following electrophoresis, the gel was cut, placed in a 21 x 31 cm polyethylene plastic tub over two sheets of 20 x 28 cm Whatman 3 MM paper, washed once briefly with dd H$_2$O, and twice for 10 minutes per wash with 300 ml 1 M sodium acetate (pH 4.0). After the last wash, the sodium acetate was removed from the tub, and the gel centered over the 3 MM paper. The freshly-prepared DBM paper was carefully placed over the gel, and any air bubbles between the gel and the DBM-paper removed. A "window frame" of saran wrap was laid over the edges of the DBM-paper to prevent contact between the wet 3 MM paper and the dry blotting paper above. Two sheets of dry Whatman 3 MM paper (9 x 13 cm) were centered over the DBM-paper, and a 3 inch thick layer of paper towels placed on top. A pane of glass was overlaid on the paper towels, and a lead brick placed on top of the glass to ensure even contact. Blotting of the DBM-paper was carried out overnight at 4 °C for a minimum of 10 hours.

Hybridization of DNA-Paper

Following blotting, the DNA-paper was removed and washed three times rapidly with dd H$_2$O, once with 50 ml of 0.3 N NaOH for 60 minutes at room temperature, three times with 100 ml dd H$_2$O rapidly, once with 25 ml 0.3 M Tris-HCl (pH 7.4) for 10 minutes at room temperature, and finally twice with 100 ml of dd H$_2$O rapidly. The DNA-paper was incubated for 60 minutes at 42 °C in a small polyethylene box containing 10 ml of pre-
hybridization buffer to minimize sporadic high background. Prehybridization buffer consists of 50% (v/v) deionized formamide; 75 mM trisodium citrate; 0.75 M NaCl; 5 times concentrated (5X) Denhardt's reagent (1966); 2.5 mM potassium phosphate (pH 7.5); 250 µg/ml calf thymus DNA; and 10 mg/ml glycine. Denhardt's reagent contains 0.2% (w/v) each of bovine serum albumin, Ficoll 400, and polyvinyl pyrrolidine. Stock solutions (25X) were treated with 0.1% (v/v) diethylpyrocarbonate to inactivate nuclease by stirring the mixture for 60 minutes prior to use. After the prehybridization step, the buffer was removed and replaced with 10 ml of hybridization buffer containing: 50% (v/v) deionized formamide; 0.75 M NaCl; 75 mM trisodium citrate; 1X Denhardt's reagent; 20 mM potassium phosphate (pH 7.5); 100 µg/ml calf thymus DNA; 10% (w/v) dextran sulfate; and $10^5$-2 x $10^5$ cpm [$^{32}$P]-labeled probe. The hybridization buffer was heated to 60 °C for 3 minutes prior to addition of the labeled probe. The probe was first denatured by heating at 97 °C for 1.5 minutes in 1.1 ml of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.1% (w/v) SDS, followed by quick cooling on ice. Hybridization was carried out at 42 °C for 24 hr. When [$^{32}$P]-labeled RNA was used as a probe, 15 µg/ml yeast RNA, 25 µg/ml poly(rA), and 0.1% (w/v) SDS, were included in the prehybridization and hybridization buffers.

After the hybridization step, the DNA-paper was washed three times (10 minutes/wash) at 42 °C with 250 ml aliquots of 0.3 M NaCl, 30 mM trisodium citrate, and 0.1% (w/v) SDS, and twice for 15 minutes per wash at 42 °C with 250 ml aliquots of 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% (w/v) SDS. The DNA-paper was air-dried, wrapped in saran wrap, and subjected to autoradiography.
Chapter III

RESULTS

This study involved analysis of the DNA products synthesized in a phosphate-buffered reconstructed reaction. The AMV reconstructed reaction can be defined as a system capable of retroviral RNA-directed DNA synthesis which utilizes the AMV 35S RNA template-tRNA\textsuperscript{Trp} primer, and purified reverse transcriptase. No other viral-associated components are used in the present reconstructed reaction. All other materials in the reaction mixture are from exogenous sources. A similar system is the oligo(dT)-primed reaction, in which oligo(dT)$_{12-18}$ is substituted for tRNA\textsuperscript{Trp} as primer. Unless otherwise specified, the term reconstructed reaction is specific for the tRNA\textsuperscript{Trp}-primed system, and not the oligo(dT)-primed system.

Optimization of AMV RNA-Directed DNA Synthesis in a Phosphate-Buffered Reconstructed Reaction

Although the AMV Tris-buffered reconstructed reaction has been optimized previously (Olsen, 1982), the phosphate-buffered system has not. The phosphate-buffered reconstructed reaction was optimized by systematic variation of each reaction component and examination of its effects on genomic-length DNA synthesis. Conditions which resulted in the maximum amount of genome-length DNA synthesis were considered to be optimal for the reconstructed reaction. Maximal amounts of genomic-length DNA were necessary to accomplish restriction mapping of the cDNA transcripts as well as to attempt to detect the presence of the second LTR.
Effect of Buffer

Previous studies of the AMV reconstructed reaction have employed the traditional Tris-HCl buffering system (Schulz et al., 1981; Olsen, 1982; Olsen and Watson, 1982). The question was asked whether a better buffering system than Tris-HCl could be found for use in the reconstructed reaction. Preliminary work demonstrated the potential of potassium phosphate as a buffer. These studies confirm the suitability of potassium phosphate as a buffer for the AMV reconstructed reaction.

A potassium phosphate (pH 8.0) concentration of 50 mM yielded the greatest percentage of genomic-length DNA synthesized in the reconstructed reaction (see Fig. 8b and 12). Concentrations of phosphate greater than 60 mM in the reaction mixture resulted in a decrease in genomic-length DNA synthesis. In the presence of 78 mM potassium phosphate (pH 8.0), the overall average size of the DNA transcripts was reduced from approximately 4.4 kb (see Fig. 7 and 11b) to 2.5-3.0 kb (see Fig. 9). A similar decrease in synthesis of genomic-length DNA was observed in reconstructed reactions buffered in 30-40 mM potassium phosphate (pH 8.0). Since the enzyme storage buffer contributes 30 mM potassium phosphate (pH 8.0) to the reconstructed reaction, the effects of phosphate concentrations less than 30 mM were not examined.

The rate of DNA synthesis in the phosphate-buffered system was faster overall than in the Tris-buffered system (Fig. 6). This increase was more pronounced in the first 40 minutes of the reaction where the rate of DNA synthesis in 50 mM potassium phosphate (pH 8.0) was 35-50% faster than in 50 mM Tris-HCl (pH 8.1). This increase gradually tapered to 10% after three hours of incubation. DNA synthesis continued at a slower
Fig. 6. Comparison of the Rates of DNA Synthesis in Reconstructed Reactions Buffered With 50 mM Tris-HCl (pH 8.1) or 50 mM Potassium Phosphate (pH 8.0). Two 150 µl reactions were incubated at 39 °C. At selected times a 20 µl aliquot was removed from each reaction and the amount of DNA synthesized was determined by the DE81 filter binding assay. Each time point represents a 2.5 µl aliquot normalized to 50 µl.

□ - 50 mM Tris-HCl (pH 8.1); 92 mM NaCl; 5 mM MgCl2; 10 mM DTT; 0.2 mM each of dATP, dGTP, and dTTP; 0.03 mM α-[32p]-dCTP (1800 cpm/pmol); 20 µg/ml 35S RNA·tRNA1rP; and saturating amounts of purified reverse transcriptase.

△ - 50 mM potassium phosphate (pH 8.0); 58 mM NaCl; 5 mM MgCl2; 10 mM DTT; 0.2 mM each of dATP, dGTP, and dTTP; 0.03 mM α-[32p]-dCTP (1800 cpm/pmol); 20 µg/ml 35S RNA·tRNA1rP; and saturating amounts of purified reverse transcriptase.
rate after three hours in both systems. The reaction was 50% complete after 25 minutes of incubation in 50 mM potassium phosphate (pH 8.0). This rate compared favorably to the 30-35 minutes required for the Tris-buffered system to reach 50% completion.

Analysis of the size of the DNA products synthesized in the phosphate-buffered system (Fig. 7) revealed that genome-length DNA was present 60 minutes after initiation of DNA synthesis. Fifteen minutes after the initiation of DNA synthesis, the largest detectable DNA species was approximately 3.0 kb in length, which increased to approximately 4.5 kb after 30 minutes of incubation. Except for accumulation of DNA corresponding to a size range of 0.5-1.5 kb, the DNA transcripts did not vary in size during the second and third hours of incubation. A general increase in the amount of DNA was observed in this period as well as accumulation of genomic DNA transcripts. Of particular interest was the detection of two discrete species of DNA 0.4 kb and approximately 0.17 kb in size in the first 30 minutes of the reaction (Fig. 7b). Both species accumulated during the remainder of the reaction but did not appear to elongate nor diminish in size. In comparison to the phosphate-buffered reaction, the Tris-buffered system synthesized DNA transcripts similar in size at a slightly slower rate. Also detected were the 0.4 kb and the 0.17 kb species, which accumulated during the reaction. The 0.4 kb species was present in lesser amounts in the Tris-buffered system, while no difference was observed in the amount of the 0.17 kb species.

Effect of 35S RNA Concentration

The concentration of 35S RNA was established that yielded the best compromise between the largest amount of DNA synthesized in a standard
Fig. 7. Rate Analysis of the Sizes of the DNA Products Synthesized in a Phosphate-Buffered Reconstructed Reaction. The DNA products synthesized in the phosphate-buffered reconstructed reaction (Fig. 6) were fractionated on alkaline 1.2% (w/v) agarose gels for 12 hr at 60 volts (constant voltage). The gel was dried, and exposed to Kodak XR film as described in Methods. Densitometer tracings were made of the resulting autoradiogram. The numerical values (in kb) refer to the relative positions of 5'-[32P]-Hind III-digested λ phage DNA markers. A - 5 min incubation; B - 15 min incubation; C - 30 min incubation; D - 60 min incubation; E - 90 min incubation; F - 120 min incubation; G - 180 min incubation.
reaction volume and the greatest percentage of DNA transcripts at or near genome-length. In general, increasing the 35S RNA concentration also increased the total amount of DNA synthesized, with a concomitant decrease in the efficiency of RNA template utilization (Table 1). At high concentrations of 35S RNA (41 μg/ml), the total yield of DNA as well as the efficiency of template utilization decreased. This was due in part to the concentration of RNA no longer being the limiting component. A 35S RNA concentration of 20 μg/ml yielded genomic DNA in acceptable quantities, and was selected as the standard 35S RNA concentration for the reconstructed reaction.

**Effect of MgCl₂**

Previous studies of the AMV Tris-buffered reconstructed reaction established a direct relationship between the extent of genomic-length DNA synthesis and the MgCl₂ concentration in the reaction mixture (Olsen, 1982). Therefore, the effects of MgCl₂ on the sizes of the DNA products in the potassium phosphate-buffered system were examined to determine if a similar relationship was present (Table 2 and Fig. 8). In contrast to the Tris-buffered system, the effect of MgCl₂ on the phosphate-buffered system was more pronounced. The greatest yield of DNA (Table 2) as well as the largest percent of genomic-length DNA was synthesized in a reaction mixture containing 5 mM MgCl₂ (Fig. 8b). Examination of the size and distribution of the DNA transcripts suggested that two distinct populations of DNA were present; a relatively large size class of average length 4.0 kb, and a smaller size class 0.1-0.5 kb in length. The large size class of DNA, which contained heterogenously sized DNA 1.5-8.0 kb, comprised 55% of the total amount of DNA synthesized. No specific sizes
Table 1. Effect of Concentration of 35S RNA·tRNA\textsuperscript{Trp} on DNA Synthesis in a Phosphate-Buffered Reconstructed Reaction.

<table>
<thead>
<tr>
<th>35S RNA·tRNA\textsuperscript{Trp} (µg/ml)</th>
<th>pmol DNA</th>
<th>pmol DNA/µg 35S RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>--</td>
</tr>
<tr>
<td>6.7</td>
<td>76.0</td>
<td>221.6</td>
</tr>
<tr>
<td>13.7</td>
<td>142.4</td>
<td>207.6</td>
</tr>
<tr>
<td>27.4</td>
<td>220.8</td>
<td>160.8</td>
</tr>
<tr>
<td>41.2</td>
<td>198.8</td>
<td>96.5</td>
</tr>
</tbody>
</table>

Purified 35S RNA·tRNA\textsuperscript{Trp} was incubated at 37 °C for 75 minutes in 50 µl reaction mixtures which contained: 50 mM potassium phosphate (pH 8.0); 5 mM MgCl\textsubscript{2}; 58 mM NaCl; 10 mM DTT; 0.2 mM each of dATP, dGTP, and dTTP; 0.03 mM \[^{3}\text{H}]-\text{dCTP} (3000 cpm/pmol); and saturating amounts of purified reverse transcriptase. Total amount of DNA synthesized was determined by the DE81 filter binding assay described in Methods.
Table 2. Effect of Magnesium Chloride on 35S RNA·tRNA<sup>Trp</sup>-Directed DNA Synthesis in a Phosphate-Buffered Reconstructed Reaction.

<table>
<thead>
<tr>
<th>Concentration MgCl&lt;sub&gt;2&lt;/sub&gt; (mM)</th>
<th>counts per minute</th>
<th>pmol DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>389,600</td>
<td>288</td>
</tr>
<tr>
<td>5</td>
<td>592,000</td>
<td>436</td>
</tr>
<tr>
<td>8</td>
<td>464,000</td>
<td>340</td>
</tr>
<tr>
<td>12</td>
<td>317,000</td>
<td>232</td>
</tr>
</tbody>
</table>

Purified AMV reverse transcriptase was incubated for 2.25 hr at 39 °C with 20 µg/ml 35S RNA·tRNA<sup>Trp</sup> and various amounts of MgCl<sub>2</sub> in 100 µl reaction mixtures which contained: 50 mM potassium phosphate (pH 8.0); 58 mM NaCl; 10 mM DTT; 0.2 mM each of dATP, dGTP, and dTTP; 0.03 mM [<sup>3</sup>H]-dCTP (1.4 Ci/mmol); 20 µg/ml 35S RNA·tRNA<sup>Trp</sup>; and saturating amounts of reverse transcriptase. Five µl aliquots from each reaction were spotted on DE81 filter paper as described in Methods, and the total amount of DNA synthesized per 100 µl determined by liquid scintillation counting. The remaining 95 µl of each reaction was extracted with phenol, and purified on a G-50 Sephadex column as described in Methods.
Fig. 8. Effect of MgCl₂ on the Sizes of the DNA Products Synthesized in a Phosphate-Buffered Reaction. The DNA products synthesized under conditions described in Table 2 were fractionated on alkaline 0.7% (w/v) agarose gels at 25 volts (constant voltage) for 5 hrs as described in Methods. Each gel was sliced, and each slice dissolved in 3 ml of Aquasol I. The total amount of DNA per slice was determined by liquid scintillation counting. The numerical values (in kb) refer to the relative positions of 5'-[32P]-Hind III-digested λ phage DNA markers. A = 3 mM MgCl₂; B = 5 mM MgCl₂; C = 8 mM MgCl₂; D = 12 mM MgCl₂.
were discernible. In contrast, the small-size class contained two relatively discrete DNA species, with approximate lengths of 0.4 kb and 0.17 kb, respectively. The species in this latter class were identical in size to those seen 2-3 hours after initiation of DNA synthesis in the phosphate-buffered reconstructed reaction (Fig. 7).

Deviation from the optimal divalent cation concentration of 5 mM MgCl₂ resulted in several changes in the DNA size distribution. Decreasing the MgCl₂ concentration to 3 mM reduced the total yield of DNA (Table 2), including DNA in both the large and small-size classes (Fig. 8a). Although the percentage of genomic-length DNA decreased to such an extent as to be undetectable, the percentage of DNA in the 1.5-8.0 kb class increased to 69% of the total amount of DNA synthesized. The percentage of DNA in the 1.5-8.0 kb range decreased to 48% of the total amount of DNA synthesized in reaction mixtures which contained 8 mM MgCl₂ (Fig. 8c), although minor amounts of genomic DNA were detectable. Increasing the MgCl₂ concentration further to 12 mM (Fig. 8d) had a deleterious effect on both the total amount of DNA synthesized (Table 2) and the percent of DNA in the large-size class (Fig. 8d). Less than 35% of the total DNA transcripts were greater than 1.5 kb, and the largest did not exceed 4.0 kb.

The relative amounts of the 0.4 and 0.17 kb species varied considerably. Reaction mixtures containing high (12 mM) or low (3 mM) concentrations of MgCl₂ did not support the synthesis of the 0.4 kb species as effectively as reaction mixtures containing either 5 or 8 mM MgCl₂. In contrast, the 0.17 kb species was a major component of all four reactions, and was the predominant species in the reaction mixture which contained
12 mM MgCl₂. Of interest was the general increase in the proportion of the 0.17 kb species compared to the total amount of DNA transcripts as the concentration of MgCl₂ was increased. This suggests that synthesis of this species was influenced by the levels of MgCl₂ present in the reaction mixture. The concentration of phosphate did not appear to affect the ratio of the two species, as the small-size class of DNA transcripts synthesized in 78 mM potassium phosphate (pH 8.0) and 5 mM MgCl₂ (Fig. 9) were similar in size and proportion to the 0.4 and 0.17 kb species synthesized in 50 mM potassium phosphate (pH 8.0) and 5 mM MgCl₂ (Fig. 8b). Similar results were observed in analysis of transcripts synthesized in concentrations of 30-40 mM potassium phosphate (pH 8.0).

Effect of Na⁺-K⁺

The effect of monovalent cation concentration on the size of the DNA transcripts synthesized in a phosphate-buffered reconstructed reaction was also examined (Table 3 and Fig. 10). The contribution of 92 mM K⁺ by the enzyme storage buffer precluded analysis of the effects of extremely low levels of Na⁺-K⁺. In view of this limitation, as well as reports by other investigators (Retzel et al., 1980; Olsen, 1982), experiments were performed varying the Na⁺-K⁺ concentrations from 100 mM to less than 200 mM, and the resulting DNA transcripts analyzed.

A Na⁺-K⁺ concentration of 150 mM in the reaction mixture yielded the greatest amount of DNA synthesized in a two hour reaction at 39 °C (Table 3). Analysis of the DNA transcripts demonstrated that this concentration also resulted in the greatest amount of genomic-length DNA, including significant quantities of the 0.4 and 0.17 kb species in roughly equimolar amounts (Fig. 10, lane 4). The bulk of the DNA tran-
Fig. 9. Size Analysis of the DNA Products Synthesized in a Reconstructed Reaction Buffered With 78 mM Potassium Phosphate (pH 8.0). The sample was fractionated on an alkaline 0.7% (w/v) agarose gel at 25 volts (constant voltage) for 5 hr, sliced, and dissolved in 3 ml of Aquasol II as described in Methods. Numerical values refer to the lengths in kb of the relative positions of 5'-'-[32p]-Hind III-digested λ phage DNA markers. The DNA products were synthesized in a 50 μl reaction mixture which contained: 78 mM potassium phosphate (pH 8.0); 5 mM MgCl2; 8 mM NaCl; 10 mM DTT; 0.2 mM each of dATP, dGTP, and dTTP; 0.03 mM [3H]-dCTP (3000 cpm/pmol); 20 μg/ml 35S RNA-tRNA1rp; and saturating amounts of purified reverse transcriptase. The reaction was incubated for 3 hr at 39 °C, and purified as described in Methods.
Table 3. Effect of Sodium Chloride on 35S RNA·tRNA<sub>Trp</sub>-Directed DNA Synthesis in a Phosphate-Buffered Reconstructed Reaction.

<table>
<thead>
<tr>
<th>Concentration NaCl (mM)</th>
<th>counts per minute</th>
<th>pmol DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>380,480</td>
<td>211.2</td>
</tr>
<tr>
<td>28</td>
<td>383,200</td>
<td>212.8</td>
</tr>
<tr>
<td>48</td>
<td>391,620</td>
<td>222.2</td>
</tr>
<tr>
<td>58</td>
<td>457,040</td>
<td>254.1</td>
</tr>
<tr>
<td>78</td>
<td>349,800</td>
<td>194.4</td>
</tr>
<tr>
<td>98</td>
<td>211,800</td>
<td>117.6</td>
</tr>
</tbody>
</table>

Purified AMV reverse transcriptase was incubated for 2.0 hr at 39 °C with various amounts of NaCl in 100 μl reaction mixtures which contained: 50 mM potassium phosphate (pH 8.0); 5 mM MgCl<sub>2</sub>; 10 mM DTT; 0.2 mM each of dATP, dGTP, and dTTP; 0.03 mM α-[<sup>32</sup>P]-dCTP (1800 cpm/pmol); 20 μg/ml 35S RNA·tRNA<sub>Trp</sub>; and saturating amounts of reverse transcriptase. The contribution of the enzyme buffer per reaction was 92 mM K<sup>+</sup>. Five μl aliquots from each reaction were spotted on DE81 filter paper as described in Methods, and the values were normalized to 100 μl reactions.
Fig. 10. Effect of NaCl on the Sizes of the DNA Products Synthesized in a Phosphate-Buffered Reconstructed Reaction. Refer to Table 3 for details of synthesis. Samples were fractionated on alkaline 1.2% (w/v) agarose gels at 50 volts (constant voltage) for 15 hr, dried and autoradiographed as described in Methods. The numerical values (in kb) refer to the relative positions of 5'-[^32P]-Hind III-digested λ phage DNA markers.

Lane 1 - 8 mM NaCl; lane 2 - 28 mM NaCl; lane 3 - 48 mM NaCl; lane 4 - 58 mM NaCl; lane 5 - 78 mM NaCl; lane 6 - 98 mM NaCl.
scripts ranged in size from 2.0 to greater than 6.0-7.0 kb. Except for a decrease of 12.6% and 16.5% in the amount of DNA synthesized in reactions containing 140 mM and 120 mM Na\(^+\)-K\(^+\), respectively, the DNA transcripts synthesized in these concentrations were similar to those synthesized in 150 mM Na\(^+\)-K\(^+\) in both size and distribution (Fig. 10, lanes 3,4). Decreasing the Na\(^+\)-K\(^+\) concentration to 100 mM, however, resulted in inhibition of genomic-length DNA synthesis (Fig. 10, lane 1). Genomic-length DNA was not detected in reactions which contained 170 mM or 190 mM Na\(^+\)-K\(^+\) (Fig. 10, lanes 5,6). The total amount of DNA synthesized was 76% and 53%, respectively, of the total yield of DNA synthesized in 150 mM Na\(^+\)-K\(^+\). Minor amounts of DNA less than 1.0 kb were also detected. The DNA transcripts synthesized in 170 mM Na\(^+\)-K\(^+\) did not exceed 5.5-6.0 kb (lane 5). Of particular interest was the detection of the 0.4 kb species but not the 0.17 kb species. In comparison, the DNA transcripts synthesized in 190 mM Na\(^+\)-K\(^+\) did not exceed 4.0 kb (lane 6). Neither the 0.4 kb nor the 0.17 kb species was detected. This was possibly due to the relatively low amount of DNA transcripts present, although the 0.17 kb species was not detected in reactions which contained 170 mM Na\(^+\)-K\(^+\). Of particular interest was the detection of several DNA species of discrete sizes greater than 0.5 kb in length in several of the reactions. These species may be due to natural stops or "stalling" by the enzyme during reverse transcription.

Effect of α-[\(^{32}\)P]-dCTP

Since α-[\(^{32}\)P]-dCTP was destined to play a major role in analysis of the cDNA transcripts in later experiments, the effects of high specific activity of α-[\(^{32}\)P]-dCTP were examined as well as different concentrations
of dCTP in the reaction mixture (Table 4). High specific activity of α-[\(^{32}\)P]-dCTP might directly affect the ability of reverse transcriptase to synthesize long DNA transcripts since the \(E_{\text{mean}}\) of \(^{32}\)P is 0.69 MeV (\(E_{\text{max}} = 1.71\) MeV) as compared to the \(E_{\text{mean}}\) of \(^{3}\)H which is 5.7 KeV (\(E_{\text{max}} = 18.6\) KeV).

A general trend noted was the observation that the higher the specific activity of α-[\(^{32}\)P]-dCTP, the lower the yield of DNA (Table 4). Increasing the concentration of dCTP from 0.03 mM to 0.1 mM while maintaining a similar specific activity (8000-8500 cpm/pmol) resulted in a 62% decrease in the total amount of DNA synthesized (Table 4, reactions 2,3). The opposite effect was observed for \(^{3}\)H-dCTP, as a slight increase in the total amount of DNA synthesized was observed when the concentration was increased from 0.03 mM to 0.1 mM while maintaining the same specific activity of \(^{3}\)H-dCTP (Table 4, reactions 7,8). Decreasing the specific activity of 0.03 mM α-[\(^{32}\)P]-dCTP to 2000 cpm/pmol or less (reactions 10,12,14) resulted in yields of DNA comparable to reaction mixtures containing 0.03 mM \(^{3}\)H-dCTP, approximately 4 pmol DNA/μl reaction mixture at 39 °C for 2 hours (Fig. 7,12). Examination of the sizes of the DNA transcripts by velocity sedimentation centrifugation revealed structural features similar to those observed using alkaline 0.7% (w/v) agarose gels (Fig. 8); the presence of two distinct classes of DNA, the large-size class (1.5-8.0 kb) and the small-size class less than 0.5 kb in length (Fig. 11). The latter was not usually resolved into separate, distinct species as observed on alkaline agarose gels. Surprisingly, the DNA transcripts synthesized in reaction mixtures which contained fresh, high specific activity (8500 cpm/pmol) α-[\(^{32}\)P]-dCTP (Fig. 11b)
Table 4. Effect of Specific Activity of Radiolabeled dCTP on 35S RNA·
tRNA^Trp-Directed DNA Synthesis in a Phosphate-Buffered Reconstructed
Reaction.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Specific Activity (cpm/pmol)</th>
<th>Isotope</th>
<th>Concentration dCTP (mM)</th>
<th>pmol DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10,200</td>
<td>[32p]</td>
<td>0.03</td>
<td>59.2a</td>
</tr>
<tr>
<td>2</td>
<td>8,500</td>
<td>[32p]</td>
<td>0.03</td>
<td>224.0</td>
</tr>
<tr>
<td>3</td>
<td>8,000</td>
<td>[32p]</td>
<td>0.10</td>
<td>84.1</td>
</tr>
<tr>
<td>4</td>
<td>8,000</td>
<td>[3H]</td>
<td>0.03</td>
<td>434.2</td>
</tr>
<tr>
<td>5</td>
<td>4,000</td>
<td>[32p]</td>
<td>0.03</td>
<td>312.5</td>
</tr>
<tr>
<td>6</td>
<td>3,000</td>
<td>[3H]</td>
<td>0.10</td>
<td>217.6b</td>
</tr>
<tr>
<td>7</td>
<td>3,000</td>
<td>[3H]</td>
<td>0.03</td>
<td>424.2</td>
</tr>
<tr>
<td>8</td>
<td>3,000</td>
<td>[3H]</td>
<td>0.10</td>
<td>465.3</td>
</tr>
<tr>
<td>9</td>
<td>3,000</td>
<td>[3H]</td>
<td>0.10</td>
<td>1440.0c</td>
</tr>
<tr>
<td>10</td>
<td>2,000</td>
<td>[32p]</td>
<td>0.03</td>
<td>376.0</td>
</tr>
<tr>
<td>11</td>
<td>2,000</td>
<td>[32p]</td>
<td>0.03</td>
<td>321.3d</td>
</tr>
<tr>
<td>12</td>
<td>1,650</td>
<td>[32p]</td>
<td>0.03</td>
<td>412.0</td>
</tr>
<tr>
<td>13</td>
<td>965</td>
<td>[3H]</td>
<td>0.03</td>
<td>517.6e</td>
</tr>
<tr>
<td>14</td>
<td>800</td>
<td>[32p]</td>
<td>0.03</td>
<td>448.0f</td>
</tr>
</tbody>
</table>

Unless otherwise stated, reactions were incubated at 39 °C for 2.5 hr in
50 μl reaction mixtures which contained: 50 mM potassium phosphate (pH 8.0);
5 mM MgCl₂; 58 mM NaCl; 10 mM DTT; 0.2 mM each of dATP, dGTP, and dTTP;
radiolabeled dCTP; 20 μg/ml 35S RNA·tRNA^Trp; and saturating amounts of
reverse transcriptase. Unless specified, radioisotope purity was greater
than 95%. The total yield of DNA was determined by the DE81 filter paper
binding assay. Each reaction was normalized to 100 μl.

a) radioisotope purity 90%
b) incubation time of 30 minutes
c) reaction included 10 μg/ml oligo(dT)₁₂-₁₈
d) isotope had decayed 3 half-lifes prior to use
e) incubation time extended to 2.5 hr
f) isotope had decayed one half-life prior to use
Fig. 11. Effect of Specific Activity of Radiolabeled dCTP on the Sizes of the DNA Products Synthesized in a Phosphate-Buffered Reconstructed Reaction. Refer to Table 4 for details of synthesis. Samples were centrifuged for 4.5 hr at 54,000 rpm in a SW60 rotor on 5-20% (w/v) alkaline sucrose gradients as described in Methods. Lengths (in kb) refer to the relative positions of 5'-[\(^{32}\)P]-Hind III-digested \(\lambda\)phage DNA markers. A - reaction 1; B - reaction 2; C - reaction 11; D - reaction 14; E - reaction 9; F - reaction 10.
were similar in size to those synthesized in reaction mixtures containing low specific activity (2000 cpm/pmol) α-[32P]-dCTP (Fig. 11f). When the specific activity was greater than 10,000 cpm/pmol, a decrease in the ratio of the large-size to small-size DNA classes was observed (Fig. 11a). The decrease in genomic-length DNA synthesis was more dramatic in reaction mixtures containing α-[32P]-dCTP that had undergone one or more half-lifes of decay (Fig. 11c,d) prior to use in the reconstructed reaction. Minor amounts of genomic-length DNA were detectable, and the proportion of DNA in the small size-class comprised a greater percentage of the total DNA products. However, the total yield of DNA was comparable to those synthesized in identical reactions using fresh α-[32P]-dCTP (Table 4, reactions 10,11). This suggested that an inhibitor was present in preparations of α-[32P]-dCTP that had undergone considerable radioactive decay. As a comparison, analysis of the DNA transcripts synthesized in an oligo(dT)-primed reaction with [3H]-dCTP revealed that the small-size DNA class comprised a much smaller percentage of the total DNA population. Plus strand synthesis was not suppressed in this reaction, and since the oligo(dT)-primed reaction has no requirement for a transcriptional jump, it is reasonable to expect that much of the small-size class of DNA was of plus polarity (Fig. 11e). Similarities between the oligo(dT)-primed reaction and the tRNA^{Trp}-primed reconstructed reactions suggest that the small-size DNA class synthesized in the latter reaction might consist of significant quantities of plus DNA, although considerable quantities might be due to cDNA that did not make the correct transcriptional jump. The increased proportion of the small-size DNA class in reactions incubated with α-[32P]-dCTP that had undergone one or more half-lifes of
radioactive decay may be due to snapback DNA. Snapback DNA (or hairpin DNA) is the result of incorrect elongation of the initial cDNA species that does not make the correct transcriptional jump.

Effect of Temperature

The incubation temperature that yielded the largest amount of DNA in a two hr reaction buffered with potassium phosphate (pH 8.0) was 39 °C (Table 5). At 37 °C, the amount of DNA synthesized under comparable conditions was 8% less. Decreasing the temperature further to 34 °C resulted in a 30% decrease compared to the amount synthesized at 39 °C. Increasing the temperature to 41 °C and 45 °C resulted in a 5% and 16% decrease, respectively, compared to that synthesized at 39 °C. The amount of genomic-length DNA did not vary noticeably in reconstructed reactions incubated at temperatures ranging from 37 °C to 41 °C. A slight decrease in the percent of genomic-length DNA was observed in reactions incubated at 34 °C and 45 °C. However, genomic-length DNA was present in all of these reactions. Since no temperature of incubation gave better results than 39 °C, this temperature was maintained in all further experiments to provide consistent results.

Comparison of the DNA Transcripts Synthesized in the Optimized Phosphate-Buffered and Tris-Buffered Reconstructed Reactions

After the various components of the phosphate-buffered system were optimized, the sizes of the DNA transcripts synthesized in 50 mM potassium phosphate (pH 8.0) were compared to those synthesized in 50 mM Tris-HCl (pH 8.1) after two hours of incubation. In terms of yield, the phosphate-buffered reaction produced 377.6 pmol of polymeric DNA as compared to 241.2 pmole synthesized in the Tris-buffered reaction. A greater percent-
Table 5. Effect of Temperature on 35S RNA•tRNA^{Trp}-Directed DNA Synthesis in a Phosphate-Buffered Reconstructed Reaction.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>pmol DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>296.8</td>
</tr>
<tr>
<td>37</td>
<td>390.0</td>
</tr>
<tr>
<td>39</td>
<td>424.2</td>
</tr>
<tr>
<td>39</td>
<td>875.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>41</td>
<td>402.9</td>
</tr>
<tr>
<td>45</td>
<td>354.9</td>
</tr>
</tbody>
</table>

Purified AMV reverse transcriptase was incubated at various temperatures for 2.0 hr in 20 µl reaction mixtures which contained: 50 mM potassium phosphate (pH 8.0); 5 mM MgCl<sub>2</sub>; 58 mM NaCl; 10 mM DTT; 0.2 mM each of dATP, dGTP, and dTTP; 0.03 mM [<sup>3</sup>H]-dCTP (3000-3300 cpm/pmol); 20 µg/ml 35S RNA•tRNA^{Trp}; and saturating amounts of purified reverse transcriptase. Total amount of polymeric DNA per reaction was determined by the DE81 filter paper binding assay as described in Methods, and normalized to 100 µl.

<sup>a</sup>25 µg/ml 35S RNA; 10 µg/ml oligo(dT)<sub>12-18</sub>
age of DNA at or near genomic-length was synthesized in the phosphate-buffered system, and the average size of the DNA transcripts, 4.0-7.0 kb, was also greater than those synthesized in the Tris-buffered reaction (Fig. 12). The 0.4 kb species was detected in both reactions, and noteworthy was the detection of several discrete species of DNA in both the phosphate-buffered and Tris-buffered systems of identical lengths. Except for the detection of increased amounts of genomic DNA, it appears that the phosphate-buffered reaction contained similar, if not identical, species of DNA compared to those found in the Tris-buffered system.

Detection of Plus DNA Synthesis in the Phosphate-Buffered Reconstructed Reaction

The question was asked whether the phosphate-buffered reconstructed reaction was capable of supporting synthesis of DNA with a plus polarity. Plus DNA has been detected in the Tris-buffered reconstructed reaction (Olsen, 1982; Olsen and Watson, 1982). Plus DNA was also detected in a phosphate-buffered, oligo(dT)-primed reaction using 35S RNA as template (Fig. 13). Analysis of the DNA transcripts synthesized in the latter reaction in the presence (Fig. 13a) or the absence (Fig. 13b) of sodium pyrophosphate (Na₄P₂O₇) by velocity sedimentation centrifugation revealed that the DNA transcripts synthesized in the presence of Na₄P₂O₇ did not contain the small-size class. Although Na₄P₂O₇ at a concentration of 4 mM had a slight inhibitory effect on reverse transcription (Fig. 5b), it also suppressed DNA-directed DNA synthesis by reverse transcriptase (Myers et al., 1977). In view of the similarities in the sizes of the small-size class of DNA synthesized in the oligo(dT)-primed reaction (Fig. 13b) and the phosphate-buffered reconstructed reaction (Fig. 8 and 14), a
Fig. 12. Comparison of the DNA Products Synthesized in a 2 Hour Optimized Phosphate-Buffered or Tris-Buffered Reconstructed Reaction. Samples containing the purified DNA products from reactions buffered with either 50 mM potassium phosphate (pH 8.0) or 50 mM Tris-HCl (pH 8.1) were fractionated on alkaline 1.2% (w/v) agarose gels for 18 hr at 50 volts (constant voltage) as described in Methods. The DNA products were synthesized in 100 µl reaction mixtures which contained: either 50 mM potassium phosphate (pH 8.0) or 50 mM Tris-HCl (pH 8.1); 58 mM or 92 mM NaCl; 5 mM MgCl$_2$; 10 mM DTT; 0.2 mM each of dATP, dGTP, and dTTP; 0.03 mM $[^3H]$-dCTP (3000 cpm/pmol); 20 µg/ml 35S RNA-tRNA$^{TP}$; and saturating amounts of purified reverse transcriptase. The reactions were incubated for 2 hr at 39 °C, and purified as described in Methods. The sizes (in kb) refer to the relative positions of 5'-[32P]-Hind III-digested λ phage DNA markers.

Lane A - 50 mM potassium phosphate (pH 8.0) and 58 mM NaCl.
Lane b - 50 mM Tris-HCl (pH 8.1) and 92 mM NaCl.
Fig. 13. Effect of Sodium Pyrophosphate on the Sizes of the DNA Products Synthesized in the Phosphate-Buffered, Oligo(dT)-Primed Reaction. Samples containing the DNA products from oligo(dT)-primed reactions synthesized in the presence (Fig. 13a) and the absence (Fig. 13b) of sodium pyrophosphate were centrifuged for 4 hr at 54,000 rpm in a SW60 rotor at 21 °C as described in Methods on alkaline 5-20% (w/v) sucrose density gradients. Each reaction consisted of 50 μl of a mixture which contained: 50 mM potassium phosphate (pH 8.0); 58 mM NaCl; 5 mM MgCl₂; 10 mM DTT; 0.2 mM each of dATP, dGTP, and dTTP; 0.03 mM [³H]-dCTP (3000 cpm/pmol); 25 μg/ml 35S RNA; 10 μg/ml oligo(dT)₁₂₋₁₈; and saturating amounts of purified reverse transcriptase. Reactions were incubated for 60 min at 39 °C and purified by phenol extraction followed by G-50 Sephadex chromatography as described in Methods.
Fig. 14. Fractionation of the DNA Products of the Optimized Phosphate-Buffered Reconstructed Reaction by Velocity Sedimentation Centrifugation. The sample was centrifuged for 4 hr at 54,000 rpm in a SW60 rotor on an alkaline 5-20% (w/v) sucrose density gradient as described in Methods. The DNA products were synthesized in a 0.5 ml reaction mixture which contained: 50 mM potassium phosphate (pH 8.0); 58 mM NaCl; 5 mM MgCl2; 10 mM DTT; 0.2 mM each of dATP, dGTP, and dTTP; 0.03 mM α-[32P]-dCTP\(^{2}\) (1330 cpm/pmol); 20 μg/ml 35S RNA·tRNA\(^{rp}\); and saturating amounts of purified reverse transcriptase. The reaction was incubated for 2 hr at 39 °C, and purified as described in Methods. Following alkaline hydrolysis overnight, the DNA products were neutralized and precipitated in ethanol at -20 °C. The numerical values refer to the relative sizes (in kb) of 5′[32P]-Hind III-digested λ phage DNA markers.
series of experiments were performed to determine the extent of plus DNA synthesis in the phosphate-buffered reconstructed reaction and to determine their sizes.

The DNA products of the phosphate-buffered reconstructed reaction synthesized in the presence and absence of actinomycin D, another reported inhibitor of plus strand synthesis, were analyzed by velocity sedimentation velocity centrifugation. Fractions containing DNA 0.2-2.0 kb in length were pooled, and hybridized with a mass excess of 35S RNA ($C_{\text{rot}} = 0.45 \text{ mol/sec} \cdot \text{mol}^{-1}$). At specific times, aliquots were removed and digested with S1 nuclease (Fig. 15). After 24 hours of hybridization, the DNA transcripts synthesized in the presence of 100 µg/ml actinomycin D were 94% resistant to S1 nuclease digestion, while the products synthesized in the absence of actinomycin D were 61% resistant. Therefore, of the remaining 33% of the DNA population unable to hybridize with 35S RNA, the majority of these should be of plus polarity. Although a minor proportion of this population might be unhybridized cDNA, it appears that a significant percentage of DNA between 0.2-2.0 kb was of plus polarity.

The sizes of DNA transcripts unable to hybridize to 35S RNA were examined (Fig. 16). The total DNA products synthesized in a phosphate-buffered reconstructed reaction were hybridized at 52 °C with a large mass excess of 35S RNA ($C_{\text{rot}} = 0.18 \text{ mol/sec} \cdot \text{mol}^{-1}$). Electrophoresis of a small aliquot of the transcripts demonstrated the presence of the two size classes of DNA seen previously; DNA transcripts 1.5-8.0 kb in length, and the small-size class less than 0.5 kb (Fig. 16a). In addition, there was a considerable proportion of DNA belonging to the intermediate-size class (0.5-1.5 kb). The unhybridized DNA was separated from the RNA-DNA hybrids
Fig. 15. Rate of Hybridization to 35S RNA of the Small-Size DNA Products Synthesized in the Phosphate Buffered Reconstructed Reaction. [3H]-labeled DNA synthesized in the optimized phosphate buffered reconstructed reaction was fractionated on alkaline 5-20% (w/v) sucrose density gradients as described in Methods. DNA 0.2-2.5 kb in length was incubated at 69 °C with a 42-fold mass excess of 35S RNA for various times. The annealing was carried out in a 75 µl reaction mixture which contained: 0.13 ng/µl [3H]-DNA, 5.7 ng/µl 35S RNA; 10 mM potassium phosphate (pH 6.1); 500 mM NaCl; and 1 mM EDTA. At selected times a 6 µl aliquot was removed and incubated for 90 min at 37 °C in 1 ml of S1 nuclease buffer and sufficient S1 nuclease to achieve complete digestion of single-stranded DNA under these conditions. The extent of resistance to S1 nuclease digestion was determined by the TCA precipitation procedure described in Methods.

△ - DNA synthesized in the presence of 100 µg/ml actinomycin D.
▽ - DNA synthesized in the absence of actinomycin D.
Fig. 16. Detection of DNA Species Unable to Hybridize With AMV 35S RNA. 
[\textsuperscript{3}H]-labeled DNA synthesized in the optimized phosphate-buffered reconstructed reaction (ref. Fig. 12 for details) were hybridized to a 40-fold mass excess of 35S RNA in 80% (v/v) formamide as described in Methods. Following hybridization, the entire reaction mixture was purified by density gradient centrifugation on 1.2 M guanidinium hydrochloride-6 M CsCl gradients at 35,000 rpm for 46 hrs at 20 °C in a SW50.1 rotor. The peak fractions containing unhybridized DNA (fractions 3-7) were desalted by G-50 Sephadex chromatography, pooled, and precipitated overnight in 70% (v/v) ethanol at -20 °C. The sample was recovered by centrifugation, and fractionated on an alkaline 0.7% (w/v) agarose gel as described in Methods.

A. Size analysis of the total DNA products prior to hybridization by alkaline 0.7% (w/v) agarose gel electrophoresis.

B. Purification of the unhybridized DNA by density gradient centrifugation.

C. Size analysis of the unhybridized DNA by alkaline 0.7% (w/v) agarose gel electrophoresis.
by equilibrium density centrifugation using 6 M CsCl-1.2 M guanidinium hydrochloride (Enea and Zinder, 1975). The procedure was modified to allow the RNA-DNA hybrids to pellet at the bottom of the tube while unhybrized single-stranded DNA migrated to an apparent density of 1.55 g/ml (Fig. 16b). Typically, the percent of DNA that migrated as single-stranded DNA comprised 15-20% of the total DNA products synthesized in the phosphate-buffered system. The unhybridized DNA was sized by alkaline agarose gel electrophoresis (Fig. 16c). The results suggested that the majority of the unhybridized single-stranded DNA was 0.1-0.7 kb in length, but transcripts up to 2.5 kb were detectable. These values generally agree with an earlier study of the DNA products synthesized in the Tris-buffered system, although plus DNA did not exceed 1.5 kb in length (Olsen, 1982). The present results suggested that plus DNA is synthesized in the phosphate-buffered reaction, and that the majority of these species belong to the small-size DNA class (0.1-0.5 kb) as observed in either alkaline agarose gels (Fig. 7,8,12) or in alkaline sucrose velocity gradients (Fig. 11,13,14). However, these results did not provide an answer as to what discrete species of plus DNA, if any, are present in the DNA population synthesized in the phosphate-buffered reconstructed reaction, since the small-size DNA class may contain immature cDNA elongation products or other artifacts of the reconstructed reaction as well as plus DNA.

Specific plus DNA species have been detected previously in the AMV RNA-directed Tris-buffered reconstructed reaction (Olsen, 1982; Olsen and Watson, 1982) using specific probes. To determine whether the phosphate-buffered system behaved similarly to the Tris-buffered system, the DNA
transcripts synthesized in the phosphate-buffered RNA-directed reconstructed reaction were examined for evidence of plus DNA synthesis using the technique of Southern blotting (Southern, 1975). Two cDNA probes were employed, cDNA, and cDNA_{rep}. cDNA_{rep} was a non-specific probe designed to detect any species of plus DNA complementary to the cDNA template. cDNA, on the other hand, was designed to detect plus DNA complementary to sequences at or near the 5'-end (3' LTR) of the cDNA template. The average size of cDNA, was 0.3 kb (+ 0.05 kb).

The DNA products of the phosphate-buffered (lane 3) and the Tris-buffered (lanes 4,5) reconstructed reactions were probed with [32p]-labeled cDNA_{rep} (Fig. 17a). In lane 3, the alkali-hydrolyzed, purified DNA products (65 ng) synthesized in the phosphate-buffered system showed evidence of heterogenous plus DNA synthesis. The plus DNA detected fell primarily into two size groups; 0.1-0.4 kb, and 0.8-2.0 kb. Minor amounts of intermediate-size plus DNA were detected (0.4-0.8 kb), but virtually no plus DNA less than 0.1 kb in length was detected. In comparison, lane 4, which contained 18 ng of the purified DNA products from the Tris-buffered reaction, showed little evidence of plus DNA synthesis. This was not surprising, as the efficiency of Southern blotting is directly proportional to the amount of sample to be detected. Lane 5, in which the DNA products of the Tris-buffered reaction were not alkali-hydrolyzed prior to electrophoresis, demonstrated that extensive amounts of fragmented 35S RNA was detected. The question of why more plus DNA was not detected in the 0.4-0.8 kb range may be due to the relative non-specificity of the cDNA_{rep} probe. Another explanation may be that, at least in the phosphate-buffered reconstructed reaction, minor quantities of plus DNA
Fig. 17. Detection of Plus DNA Synthesis in a Phosphate-Buffered Reconstructed Reaction By Southern Blotting. $[^{3}H]$-labeled DNA synthesized in standard reconstructed reactions (refer to Fig. 12) buffered in either 50 mM potassium phosphate (pH 8.0) or 50 mM Tris-HCl (pH 8.1) were alkali-hydrolyzed, and purified as described in Methods. The samples were fractionated on alkaline 2.5% (w/v) agarose gels for 6 hr at 150 mA (constant current). The DNA products were transferred to DBM-paper as described in Methods and hybridized to $[^{32}P]$-labeled cDNA probes.

A. cDNA$_{rep}$ probe. Lane 1 - 5'-$[^{32}P]$-Hind III-digested $\lambda$ phage DNA markers; lane 2 - 5'-$[^{32}P]$-Hae III-digested $\phi$X-174 phage DNA markers; lane 3 - 65 ng DNA from the phosphate-buffered reaction; lane 4 - 18 ng DNA from the Tris-buffered reaction; lane 5 - 25 ng DNA from the Tris-buffered reaction (no alkali hydrolysis).

B. cDNA$_{3'}$ probe. Lane 1 - 5'-$[^{32}P]$-Hind III-digested $\lambda$ phage DNA markers; lane 2 - 143 ng DNA from the phosphate-buffered reaction; lane 3 - 86 ng DNA from the Tris-buffered reaction; lane 4 - 5'-$[^{32}P]$-Hae III-digested $\phi$X-174 phage DNA markers.
corresponding to this size range were actually synthesized.

When the specific probe cDNA$_3$ was used, several discrete species of plus DNA were detected (Fig. 17b). Specific plus DNA species detected by cDNA$_3$, that had been synthesized in the phosphate-buffered reconstructed reaction (lane 2) corresponded to sizes of 0.4 kb, 0.3 kb, and 0.17 kb. Plus DNA$_{400}$ and plus DNA$_{300}$ (mass ratio 4:1) have been detected previously in studies of the AMV Tris-buffered reconstructed reaction (Olsen, 1982, Olsen and Watson, 1982), as well as a 0.17 kb hairpin specie. These species appeared shortly after initiation of DNA synthesis in the Tris-buffered system. DNA transcripts 0.4 kb and 0.17 kb were also detected shortly after initiation of DNA synthesis in the phosphate-buffered system (Fig. 7). No other plus DNA species greater than 0.4 kb were detected in the phosphate-buffered reconstructed reaction with cDNA$_3$.

Since the AMV LTR is 385 nucleotides in length (Rushlow et al., 1982), plus DNA$_{400}$ may represent the plus DNA species thought to be involved in the second transcriptional jump (Fig. 2). The results of Southern blotting of the DNA species synthesized in the phosphate-buffered system suggest that plus DNA synthesis is discontinuous since different species were detected with different probes. These three species of plus DNA were also detected in the Tris-buffered reconstructed reaction (lane 3). Plus DNA greater than 0.4 kb was also not detected in the Tris-buffered system with cDNA$_3$.

**Detection of Other Small-Size DNA Species in the Phosphate-Buffered Reconstructed Reaction**

In view of the considerable amount of DNA contained in the secondary, small-size DNA class less than 0.5 kb in length (Fig. 13), this group
was examined in further detail to attempt to detect other species in addition to plus DNA$_{400}$, plus DNA$_{300}$, and the 0.17 kb species. The DNA products that had been synthesized in the phosphate-buffered reconstructed reaction were fractionated by velocity sedimentation centrifugation (Fig. 14). Fractions containing DNA less than 1.3 kb in length (fractions 20-25) were fractionated in separate lanes on a 4.8% (w/v) polyacrylamide-7 M urea gel (Fig. 18). The results demonstrated the existence of several discrete DNA species within this class of small-size DNA. Of these species, three were of plus polarity; plus DNA$_{400}$ (fractions 20-24), plus DNA$_{300}$ (fractions 21-24), and an unknown species, x (fractions 22-25). Although not characterized further, x, approximately 0.09 kb, has been detected previously in the Tris-buffered reconstructed reaction (Olsen, 1982; Olsen and Watson, 1982). It was reported that x did not hybridize with 35S RNA, but neither could x be detected using Southern blotting with cDNA$_{rep}$, cDNA$_{3'}$, or cDNA$_{5'}^{100}$ probes. Other identifiable species included cDNA$_{5'}^{100}$ (fractions 23-25), and hairpin DNA (fractions 22-25). In this gel system, which was only partly denaturing, hp DNA migrated with a size corresponding to 75-80 nucleotides although it was actually 170-175 nucleotides in length. Hairpin DNA, x, and plus DNA$_{400}$ comprised the majority of discrete species less than 0.5 kb in length after 2 hours of reverse transcription in the phosphate-buffered system. In contrast, cDNA$_{5'}^{100}$ and plus DNA$_{300}$ each represented less than 8% of the DNA population less than 0.5 kb. Other minor species of unknown polarity were detected (fractions 23-25) which possibly represent immature elongation products of cDNA$_{5'}^{100}$ or heterogenously-sized plus DNA.
Fig. 18. Analysis of the Small-Size DNA Species Synthesized in a Phosphate-Buffered Reconstructed Reaction. Alkalihydrolyzed DNA synthesized in a phosphate-buffered reconstructed reaction was fractionated on an alkaline 5-20% (w/v) sucrose density gradient for 4 hr at 54,000 rpm at 21 °C in a SW60 rotor (Fig. 13). DNA-containing fractions less than 1.3 kb in length (Fig. 13, fractions 20-25) were neutralized, and precipitated overnight in 70% (v/v) ethanol at -20 °C as described in Methods. Following recovery by centrifugation, the samples were fractionated in separate lanes on a 4.8% (w/v) polyacrylamide-7 M urea gel at 12 mA for 2.8 hr, dried, and autoradiographed. (a) 5'-[32P]-Hae III-φX-174 phage DNA markers; (b) fraction 20; (c) fraction 21; (d) fraction 22; (e) fraction 23; (f) fraction 24; (g) fraction 25; (h) same as (a).
Development of a Restriction Map of the AMV Complex cDNA Transcripts Synthesized in a Phosphate-Buffered Reconstructed Reaction

A restriction map of the AMV complex based upon the cDNA transcripts synthesized in the phosphate-buffered reconstructed reaction was developed to demonstrate that the reconstructed reaction was capable of synthesis of DNA biochemically indistinguishable from those found either in infected cells or in the endogenous reaction. Also, restriction mapping of cDNA transcripts could provide a means by which specific cDNA probes could be generated. Early attempts to produce a restriction map utilizing cDNA-RNA hybrids (Malloy and Symons, 1980) were not successful. Therefore restriction mapping was performed using \(^{32}\text{P}\)-cDNA-\(^{3}\text{H}\)-plus DNA duplexes as described in Methods.

Synthesis of Plus DNA Using cDNA as Template and S1 Nuclease-Digested Calf Thymus DNA as Primers

Second strand synthesis was required to provide double-stranded DNA (ds DNA) substrates for restriction mapping of the cDNA template. The reaction conditions were optimized as described in Methods to ensure that as many of the cDNA template restriction sites were converted in ds DNA as possible.

The highest yield of plus DNA was achieved with 350 \(\mu\text{g/ml}\) calf thymus DNA primers, and allowing the primer-template mixture to hybridize for 30 minutes prior to addition of the restriction endonuclease. Under these conditions, typical yields of plus DNA ranged from 70% to greater than 90% of the amount of available cDNA template (mass ratios of 0.7 and greater than 0.9, respectively). The extent of DNA synthesis due to calf thymus DNA acting as both template and primer was less than
2% of the total amount of plus DNA synthesis.

A side reaction consistently observed was an apparent extension of the cDNA template both in the presence and absence of exogenous primers (Fig. 19). While not understood, the mechanism most likely involved in cDNA template extension is the formation of hairpins at the 3'-end of the nascent cDNA template upon termination of reverse transcription, allowing the cDNA transcript to act as its own template and primer. Heating and quick-cooling of the template-primer mixture prior to hybridization reduced the amount of plus DNA synthesis by this mechanism from 29% to less than 10% of the total amount of plus synthesis (Fig. 19b).

Relatively short strands of plus DNA were observed with cDNA templates of various sizes. Use of cDNA templates 3.3-3.9 kb resulted in synthesis of plus DNA species 0.3-2.0 kb (mass ratio 0.85) (Fig. 19a,b). Of interest was the detection of several discrete species of plus DNA, similar to cDNA transcripts synthesized in the phosphate-buffered reconstructed reaction (Fig. 12). The size of the plus DNA transcripts synthesized on shorter cDNA templates of 1.1-1.4 kb and 0.5-0.75 kb were correspondingly shorter in size, approximately 0.35-0.45 kb (Fig. 19c-f). In several experiments the yield of plus DNA exceeded the amount of available cDNA template, which suggested that synthesis of plus DNA might involve strand displacement of pre-existing plus DNA transcripts hydrogen bonded to the cDNA template. Although not quantified, plus DNA transcripts were less resistant to S1 nuclease digestion when the yield of plus DNA was greater than 100% of the available amount of cDNA template, and relatively insensitive to digestion when the percent yield was less than 90%. Except for the extension of cDNA template, the plus DNA did not approach
Fig. 19. Analysis of the Plus DNA Species Synthesized Using a Presized cDNA Template and SI Nuclease-Digested Calf Thymus DNA Primers. Unless otherwise specified, plus DNA was synthesized in 50 μl reaction mixtures which contained: 50 mM Tris-HCl (pH 8.1); 8 mM MgCl₂; 40 mM NaCl; 10 mM DTT; 0.5 mM each of dATP, dGTP, and dTTP; 0.1 mM α-[32P]-dCTP (1800 cpm/pmol); presized [3H]-cDNA template (1.6-2.0 ng/μl); 0.35 μg/μl SI nuclease-digested calf thymus DNA primers; and saturating amounts of purified reverse transcriptase. The reactions were incubated at 39 °C for 90 min, and purified as described in Methods. Samples were fractionated on alkaline 1.2% (w/v) agarose gels for 22 hr at 45 volts (constant voltage), dried, and autoradiographed. Enhanced densitometer tracings of the resulting autoradiograms were made. Numerical values refer to the lengths (in kb) of 5′-[32P]-Hind III-digested λ phage DNA markers and 5′-[32P]-Hae III-digested φX-174 phage DNA markers. A - [3H]-cDNA template 3.5-3.9 kb; B - [3H]-cDNA template 3.3-3.7 kb; C - [32P]-cDNA template 1.0-1.5 kb, [3H]-plus DNA; D - [3H]-cDNA template 1.1-1.4 kb; E - [32P]-cDNA template 0.5-0.7 kb, [3H]-plus DNA; F - [3H]-cDNA template 0.5-0.7 kb. B-F were heated and quick-cooled as described in Methods prior to addition of enzyme.
a size corresponding to the length of the original cDNA template.

**Kpn I**

*Kpn I* was the first restriction endonuclease observed to produce cleavage products of the double-stranded DNA (Fig. 20, 26). Digestion of duplex DNA of approximate length 3.4-5.4 kb by *Kpn I* resulted in the appearance of a 2.3 kb fragment (A) (Fig. 20a,b). No other distinct fragments were detected due to the heterogenous size of the cDNA template. In a second experiment, digestion of ds DNA 3.9-4.7 kb in length yielded a similar fragment (A) 2.4 kb in size, as well as a heterogenously sized fragment (B) approximately 1.9 kb (Fig. 20c,d, Fig. 26). In order to confirm the correct restriction site for *Kpn I* on the cDNA template, digestion of duplex DNA slightly smaller in length generated a 2.35 kb fragment (A) and a secondary fragment (B) approximately 1.7 kb (Fig. 20e,f). The results of these experiments suggested that a single *Kpn I* restriction site was located 2.35 kb from the 5'-end of the cDNA transcripts. Comparison with previously published maps (Bergmann et al., 1981; Baluda et al., 1983) indicated that the MAV-1 genome also contained a single *Kpn I* restriction site located 2.3 kb from the 5'-end of the cDNA template (3' LTR).

**Eco RI**

Digestion of cDNA-plus DNA duplexes of various lengths with the restriction endonuclease *Eco RI* produced evidence for multiple *Eco RI* restriction sites located on the AMV complex cDNA transcripts (Fig. 21, 26). Present in all digests was a 1.27 kb fragment (A), which suggested an *Eco RI* restriction site was located 1.27 kb from the 5'-end of the cDNA transcripts (Fig. 21). Digestion of large cDNA-plus DNA duplexes
Fig. 20. Restriction Mapping of Presized Duplex DNA With Kpn I. Samples containing presized cDNA plus DNA duplexes synthesized as described in Methods were digested overnight at 37 °C in 50 μl reaction mixtures which contained: 6 mM MgCl₂; 6 mM NaCl; 6 mM Tris-HCl (pH 7.4); 6 mM β-mercaptoethanol; 100 μg/ml calf thymus DNA; and 9-18 units of Kpn I. Following purification, the samples were fractionated on alkaliine 1.2% (w/v) agarose gels at 50 volts for 16-20 hrs, dried, and autoradiographed as described in Methods. Enhanced densitometer tracings were made of the resulting autoradiograms. Numerical values refer to the lengths (in kb) of 5'-[³²P]-Hind III-digested λ phage DNA markers and φX-174 phage DNA markers.

(a) 8.6 ng undigested duplex DNA (mass ratio 0.29) 3.4-5.4 kb avg size; (b) same as (a) but digested with 9 units of Kpn I; (c) 7.5 ng undigested duplex DNA (mass ratio 0.54) 4.0-4.6 kb avg size; (d) same as (c) but digested with 18 units of Kpn I; (e) 3.3 ng undigested duplex DNA (mass ratio 0.6) 3.8-4.4 kb avg size; (f) same as (e) but digested with 18 units of Kpn I.
Fig. 21. Restriction Mapping of Presized Duplex DNA With Eco R1. Samples containing presized duplex DNA were digested overnight at 37 °C in 50 μl reaction mixtures which contained: 100 mM Tris-HCl (pH 7.5); 50 mM NaCl; 5 mM MgCl₂; 100 μg/ml calf thymus DNA; and 16 units of Eco R1. Following purification, the samples were fractionated on alkaline 1.2% (w/v) agarose gels for 16-20 hr at 50 volts, dried, and autoradiographed as described in Methods. Enhanced densitometer tracings of the resulting autoradiograms were made. Refer to Fig. 20 for DNA marker lengths.

(a) 3.4 ng of undigested duplex DNA (mass ratio 0.72) 4.2-5.0 kb avg size; (b) same as (a) but digested with Eco R1; (c) 2.8 ng of undigested duplex DNA (mass ratio 0.65) 4.0-5.0 kb avg size; (d) same as (c) but digested with Eco R1; (e) 3.9 ng of undigested duplex DNA (mass ratio 0.59) 3.1-4.1 kb avg size; (f) same as (e) but digested with Eco R1; (g) 3.8 ng undigested duplex DNA 2.8-3.4 kb avg size (mass ratio 0.86); (h) same as (g) but digested with Eco R1.
(Fig. 21a-d,26) also resulted in detection of two restriction fragments 3.9 kb (C) and 3.2 kb (B) in length which were not observed in digests of smaller duplexes (Fig. 21e-h). This suggested that these latter restriction fragments were located internally with respect to the 5'-end of the cDNA transcripts. However, the summation of these three fragments exceeded the original sizes of the control cDNA transcripts. In addition, a minor restriction fragment (D) 1.35 kb in length was detected in digests of smaller duplexes (Fig. 21e-h), but not in larger duplexes (Fig. 21a-d). None of these fragments appeared to be the result of incomplete digestion as no one fragment was the summation of two or more smaller fragments. It is of interest to note that the 1.27 kb and 3.9 kb fragments corresponded closely with the MAV-1 restriction map (Fig. 4), while the 1.35 kb and 3.2 kb species were identical to those reported for AMV (Baluda et al., 1983). The results of these experiments suggested that at least two, and probably four, Eco R1 restriction sites are located in the 5'-half of genomic cDNA transcripts synthesized in the phosphate-buffered reconstructed reaction with AMV complex 35S RNA.

**Bam H1**

Digestion of cDNA-plus DNA duplexes 1.9-3.0 kb in length (Fig. 22) with the restriction endonuclease **Bam H1** resulted in generation of a primary restriction fragment (A) 1.86 kb in length, and heterogenously sized secondary restriction fragments. The 1.86 kb fragment did not vary in size. Digestion of duplex DNA of average length 1.6 kb with 20 units of **Bam H1** resulted in no detectable change in the size of the duplex DNA (Fig. 22g,h). The results of these experiments suggest a single **Bam H1** restriction site is located 1.86 kb from the 5'-end of the cDNA.
Fig. 22. Restriction Mapping of Presized Duplex DNA With Bam H1. Samples containing presized DNA duplexes were digested overnight at 37 °C in 50 µl reaction mixtures which contained: 150 mM NaCl; 6 mM Tris-HCl (pH 7.9); 6 mM MgCl₂; 100 µg/ml calf thymus DNA; and 20 units of Bam H1. Following purification, the samples were fractionated on alkaline 1.2% (w/v) agarose gels at 50 volts for 16-20 hr, dried, and autoradiographed as described in Methods. Enhanced densitometer tracings of the resulting autoradiograms were made. Refer to Fig. 20 for marker details.

(a) 2.1 ng undigested duplex DNA (mass ratio 0.79) 2.6-2.9 kb avg size; (b) same as (a) but digested with Bam H1; (c) 2.0 ng undigested duplex DNA (mass ratio 0.71) 2.0-2.5 kb avg size; (d) same as (c) but digested with Bam H1; (e) 1.9 ng undigested DNA (mass ratio 0.9) 1.7-2.1 kb avg size; (f) same as (e) but digested with Bam H1; (g) 2.9 ng undigested duplex DNA (mass ratio 0.81) 1.4-1.8 kb avg size; (h) same as (g) but digested with Bam H1.
template. Comparison to previously published restriction maps of MAV-1 indicates that a Bam HI restriction site is located 1.86 kb from the 5'-end of the cDNA template (3' LTR) (Bergmann et al., 1981; Baluda et al., 1983).

Hgi A1

Digestion of a cDNA-plus DNA duplex of average length 3.4 kb with Hgi A1 resulted in detection of three restriction fragments (Fig. 23a,b). Although the summation of these fragments exceeded the original size of the duplex DNA by almost two-fold, the 3.2 kb fragment (A) may have represented the incomplete digestion product of the 2.6 kb (B) and the 0.51 kb (C) fragments. Analysis of the digestion products of smaller duplexes (Fig. 23c-f) revealed that the 0.51 kb fragment did not change in size, and the 2.6 kb and 3.2 kb fragments were not detected. These results provided evidence for at least two Hgi A1 restriction sites located 3.2 kb and 0.51 kb from the 5'-end of the AMV complex cDNA transcripts. This restriction endonuclease has not been used previously to map the AMV, MAV-1 or MAV-2 genomes, therefore, no comparison can be made. Because MAV-1 is the predominant species in the AMV complex (Bergmann et al., 1980), these sites detected are probably located on the MAV-1 cDNA transcripts and possibly the 0.51 kb fragment is common to all three species.

Hind III

Two Hind III restriction sites on the cDNA template were detected (Fig. 24). Restriction digestion of duplex DNA of various lengths with Hind III resulted in the detection of a 1.02-1.1 kb fragment (A) (Fig. 24a-h). However, analysis of the digestion products generated in two of these experiments resulted in the detection of a second fragment (B).
Fig. 23. Restriction Mapping of Presized Duplex DNA With Hqi Al. Samples containing presized duplex DNA were digested overnight at 37 °C in 50 µl reaction mixtures which contained: 200 mM NaCl; 10 mM Tris-HCl (pH 8.0); 10 mM MgCl₂; 10 mM β-mercaptoethanol; 100 µg/ml calf thymus DNA; and 20 units of Hqi Al. Samples were fractionated on alkaline 1.2% (w/v) agarose gels for 16-20 hr at 50 volts, dried, and autoradiographed as described in Methods. Refer to Fig. 20 for explanation of DNA marker sizes. (a) 1.7 ng undigested duplex DNA (mass ratio 0.85) 3.2-3.6 kb avg size; (b) same as (a) but digested with Hqi Al; (c) 2.0 ng undigested duplex DNA (mass ratio 0.71) 1.9-2.5 kb avg size; (d) same as (c) but digested with Hqi Al; (e) 1.9 ng undigested duplex DNA (mass ratio 0.92) 1.6-2.2 kb avg size; (f) same as (e) but digested with Hqi Al.
Fig. 24. Restriction Mapping of Presized Duplex DNA With Hind III. Samples containing presized DNA duplexes were digested overnight at 37 °C in 50 μl reaction mixtures which contained: 60 mM NaCl; 7 mM MgCl₂; 7 mM Tris-HCl (pH 7.5); 100 μg/ml calf thymus DNA; and 20 units of Hind III. Following purification, samples were fractionated on alkaline 1.2% (w/v) agarose gels at 50 volts for 16-20 hr, dried, and autoradiographed as described in Methods. Enhanced densitometer tracings of the resulting autoradiograms were made. Refer to Fig. 20 for explanation of markers. (a) 1.6 ng undigested duplex DNA (mass ratio 0.85) 3.2-3.8 kb avg size; (b) same as (a) but digested with Hind III; (c) 2.1 ng undigested duplex DNA (mass ratio 0.79) 2.6-3.0 kb avg size; (d) same as (c) but digested with Hind III; (e) 2.5 ng of undigested duplex DNA (mass ratio 0.77) 2.1-2.5 kb avg size; (f) same as (e) but digested with Hind III; (g) 4.2 ng undigested DNA duplex (mass ratio 0.54) 1.6-2.1 kb avg size; (h) same as (g) but digested with Hind III.
0.2-0.25 kb in length (Fig. 24d,h). This fragment was not detected in digests of duplex DNA 3.2-3.8 kb and 2.1-2.5 kb (Fig. 24b,f). The results of these experiments suggested that there are two Hind III restriction sites near the 5'-end of the cDNA transcripts, with cleavage products of 0.2-0.25 kb and 1.05 kb. Although the data did not suggest the order of these two fragments, comparison with the MAV-1 restriction map (Fig. 4) indicated that there are two sites located 0.2 kb and 1.3 kb from the 5'-end of the MAV-1 cDNA template (Baluda et al., 1983).

Xho I

Digestion of cDNA-plus DNA duplexes 2.5 kb and larger in length with Xho I resulted in detection of restriction fragments corresponding to lengths 0.47 kb (A), 1.56 kb (B), 2.1 kb (C), and 1.72 kb (D) (Fig. 25a-d). The 1.72 kb fragment appeared as a shoulder of the 1.56 kb fragment and the ratio of the amounts of the 1.56 kb and 1.72 kb species was approximately 2:1. Neither the 2.1 kb nor the 1.72 kb fragments were detected when duplexes smaller than 2.5 kb were digested with Xho I (Fig. 25e,f). The 2.1 kb fragment may have been the result of incomplete digestion of duplex DNA. Comparison with previously published maps of MAV-1 (Bergmann et al., 1981; Baluda et al., 1983) indicated that the MAV-1 genome contains Xho I restriction sites located 2.1 kb and 0.47 kb from the 5'-end of the cDNA template (Fig. 4). Conversely, the 1.72 kb fragment did not match either the known MAV-1 or AMV restriction maps (Baluda et al., 1983). Mapping data for MAV-2 is incomplete, however, and this fragment may represent a restriction site located either 1.72 kb or 2.3 kb from the 5'-end of the MAV-2 cDNA template. The AMV genome also contains a single restriction site specific for Xho I located 0.47 kb
Fig. 25. Restriction Mapping of Presized Duplex DNA With Xho I. Samples containing presized duplex DNA were digested overnight at 37°C in 50 μl reaction mixtures which contained: 150 mM NaCl; 6 mM Tris-HCl (pH 7.9); 6 mM MgCl₂; 6 mM β-mercaptoethanol; 100 μg/ml calf thymus DNA; and 20 units of Xho I. Following purification, the samples were fractionated on alkaline 1.2% (w/v) agarose gels for 16-20 hr, dried, and autoradiographed as described in Methods. Enhanced densitometer tracings of the resulting autoradiograms were made. Refer to Fig. 20 for explanation of DNA marker sizes.

(a) 1.9 ng of undigested duplex DNA (mass ratio 0.86) 3.3-3.7 kb avg size; (b) same as (a) but digested with Xho I; (c) 3.2 ng of undigested duplex DNA (mass ratio 0.69) 2.5-3.1 kb avg size; (d) same as (c) but digested with Xho I; (e) 5.5 ng of undigested duplex DNA (mass ratio 0.55) 2.0-2.4 kb avg size; (f) same as (e) but digested with Xho I.
Fig. 26. Restriction Mapping of Presized cDNA-Plus DNA Duplexes With Various Restriction Endonucleases. Autoradiograms of selected restriction digests of presized duplex DNA were taken. Refer to Fig. 20-21 for the reaction conditions. Lanes 1,8,9 - 5'-[32P]-Hind III-digested λ phage DNA markers; lane 2 - Fig. 21a; lane 3 - Fig. 21b; lane 4 - Fig. 21c; lane 5 - Fig. 21d; lane 6 - Fig. 20c; lane 7 - Fig. 20d; lane 10 - 5'-[32P]-Hae III-digested φX-174 phage DNA markers; lane 11 - Fig. 21e; lane 12 - Fig. 21d.
from the 5'-end of the AMV cDNA transcripts, but does not contain any additional, known Xho I restriction sites in the 5'-half of the AMV cDNA template.

The results of the mapping studies are summarized in Table 6 and also presented in the form of a map (Fig. 27). Except for the Xho I 1.72 kb fragment, the mapping results obtained using cDNA transcripts synthesized in the phosphate-buffered reconstructed reaction are either in close or exact agreement with previously published maps of MAV-1, MAV-2, and AMV (Bergmann et al., 1980, 1981; Baluda et al., 1983). This suggests that the cDNA products synthesized in the phosphate-buffered reconstructed reaction are closely related biochemically to those species found in vivo and in the endogenous reaction. Furthermore, the results of these studies demonstrate a potential method by which specific cDNA sequences can be generated for possible use as cDNA probes.

Detection of the Second Long Terminal Redundancy (LTR)

The question of whether the second LTR (5' LTR) is present is genomic cDNA transcripts synthesized in the phosphate-buffered reconstructed reaction was addressed. Although the second LTR has not been detected in either the AMV endogenous or reconstructed reactions (unpublished observations), the Moloney murine leukemia virus endogenous reaction has been shown to be capable of supporting synthesis of the second LTR via a putative second transcriptional jump (Gilboa et al., 1979). Three requirements must be met to demonstrate the presence or absence of the second LTR in genomic cDNA transcripts by Southern blotting; adequate amounts of genomic cDNA, an efficient method of distinguishing between both LTRs, and a LTR-specific probe.
Table 6. Summary of Results from Restriction Mapping of cDNA Transcripts Synthesized in a Phosphate-Buffered Reconstructed Reaction.

<table>
<thead>
<tr>
<th>Restriction Endonuclease</th>
<th>Average cDNA Template Size (kb)</th>
<th>Cleavage Products (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bam HI</td>
<td>2.6 ± 0.3</td>
<td>1.85, 0.6-1.0</td>
</tr>
<tr>
<td></td>
<td>2.2 ± 0.25</td>
<td>1.86, less than 0.6</td>
</tr>
<tr>
<td></td>
<td>1.9 ± 0.25</td>
<td>1.86</td>
</tr>
<tr>
<td></td>
<td>1.6 ± 0.25</td>
<td>- - -</td>
</tr>
<tr>
<td>Eco RI</td>
<td>4.6 ± 0.4</td>
<td>1.27, 3.85, 3.2</td>
</tr>
<tr>
<td></td>
<td>4.5 ± 0.5</td>
<td>1.3, 3.9, 2.9-3.2</td>
</tr>
<tr>
<td></td>
<td>3.6 ± 0.5</td>
<td>1.27, 1.34, 2.0-2.3</td>
</tr>
<tr>
<td></td>
<td>3.1 ± 0.25</td>
<td>1.27, 1.35, 1.7-2.0</td>
</tr>
<tr>
<td>HgiAl</td>
<td>3.4 ± 0.2</td>
<td>3.2, 2.6, 0.51</td>
</tr>
<tr>
<td></td>
<td>2.2 ± 0.25</td>
<td>1.6-1.8, 0.51</td>
</tr>
<tr>
<td></td>
<td>1.9 ± 0.25</td>
<td>1.3-1.4, 0.54</td>
</tr>
<tr>
<td>Hind III</td>
<td>3.5 ± 0.3</td>
<td>1.05, 1.9-2.3</td>
</tr>
<tr>
<td></td>
<td>2.8 ± 0.2</td>
<td>1.05, 0.2-0.25, 1.3-1.9</td>
</tr>
<tr>
<td></td>
<td>2.3 ± 0.15</td>
<td>1.1, 1.1-1.3</td>
</tr>
<tr>
<td></td>
<td>1.8 ± 0.25</td>
<td>1.02, 0.2-0.25</td>
</tr>
<tr>
<td>Kpn I</td>
<td>4.4 ± 1.0</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>4.3 ± 0.25</td>
<td>2.4, 1.7-2.1</td>
</tr>
<tr>
<td></td>
<td>4.1 ± 0.3</td>
<td>2.35, 1.7-1.85</td>
</tr>
<tr>
<td>Xho I</td>
<td>3.5 ± 0.2</td>
<td>1.56, 0.47, 2.1, 1.72</td>
</tr>
<tr>
<td></td>
<td>2.8 ± 0.3</td>
<td>1.56, 0.47, 2.1, 1.72</td>
</tr>
<tr>
<td></td>
<td>2.2 ± 0.25</td>
<td>1.56, 0.46</td>
</tr>
</tbody>
</table>
Fig. 27. Restriction Map of the AMV Complex Genome. Refer to Table 6 for summary of restriction fragments generated in the restriction mapping experiments. Question marks refer to possible sites mapped.

A. Restriction map based on total cDNA transcripts.
B. Restriction map based on the MAV-1 cDNA genome
C. Restriction map based on the AMV cDNA genome.
Synthesis of cDNA was performed as described in Methods in two separate reactions (1.0 ml and 0.75 ml) using the optimized phosphate-buffered reaction conditions, except that $[^3H]$-dCTP was substituted for $\alpha$-[32P]-dCTP. The incubation time was increased to 2.5 hr to maximize synthesis of genomic DNA, with total yields of 1.31 and 0.99 µg of acid insoluble nucleic acid. Analysis of the sizes of the DNA products by sedimentation velocity centrifugation revealed a relatively poor yield of cDNA greater than 5.0 kb in length (4.4% and 7.9% of the total DNA products synthesized), as well as an increased amount of DNA species 0.5-2.5 kb in length (Fig. 28a). cDNA greater than 5 kb was pooled, and a small aliquot was fractionated on an alkaline 0.7% (w/v) agarose gel (Fig. 28b). Examination of the size of the pooled cDNA indicated that 90% was greater than 5 kb, with the remaining 10% greater than 4.0-4.4 kb. Of this amount of cDNA greater than 5 kb, less than 20-25% were probably at or near genomic-length.

Synthesis of the plus strand was performed essentially as described in Methods, except that the reaction was allowed to incubate for 2 hours instead of 90 minutes. The extent of plus DNA synthesis was 55% of the mass of the available cDNA template (26.5 ng plus DNA).

The source for the LTR-specific probe was AMV 8-10S poly(A)$^+$ RNA. Plus DNA$_{400}$ was not used because it was not available in sufficient quantities. Following end labeling at its 5'-ends with $\gamma$-[32P]-ATP, the RNA probe was analyzed on a 4.8% (w/v) polyacrylamide-7 M urea gel (Fig. 29a). The results demonstrated that the probe was heterogenous in size, and ranged from less than 50 nucleotides to greater than 500. To ensure the specificity of the probe, it was fractionated by sedimentation
Fig. 28. Selection of Genomic cDNA Transcripts for Detection of the Second LTR. The [3H]-DNA products synthesized in the optimized phosphate-buffered reconstructed reaction (ref. Fig. 12) were alkali-hydrolyzed, and purified as described in Methods. The sample was fractionated by velocity sedimentation centrifugation (Fig. 28a) on alkaline 5-20% (w/v) sucrose density gradients as described in Methods. DNA greater than 5.0 kb in length (fractions 9-13) were pooled, neutralized, and precipitated with ethanol. A small aliquot was fractionated on an alkaline 0.7% (w/v) agarose gel for 5 hr at 25 volts (constant voltage) to determine the size of the pooled cDNA (Fig. 28b). The gel was sliced and each slice dissolved in 3 ml of Aquasol II. The numerical values refer to the lengths (in kb) of 5'-[32p]-Hind III-digested λ phage DNA markers.
Fig. 29. Analysis of the Size of the Sub 8-10S RNA Probe Specific for the LTR. A small aliquot of the 5'-\([32P]\)-labeled sub 8-10S poly(A)+ RNA probe was fractionated on a 4.8% (w/v) polyacrylamide-7 M urea gel at 150 mA (constant current) for 2.8 hr as described in Methods (Fig. 29a). The remainder of the labeled sub 8-10S RNA probe (20 µg) was centrifuged on a 4.4 ml 10-30% (v/v) glycerol gradient for 24 hr at 54,000 rpm at 20 °C. The leading fractions (front slope of the sub 8-10S RNA peak) were pooled, and precipitated with ethanol at -20 °C. A small aliquot of the purified probe was fractionated on a 4.8% (w/v) polyacrylamide-7 M urea gel (Fig. 29b) at 150 mA (constant current) for 2.8 hr. The gels were dried, autoradiographed, and densitometer tracings of the resulting autoradiograms were made. The numerical values refer to the lengths (in kb) of 5'-\([32P]\)-Hae III-digested ϕX-174 phage DNA markers.
velocity centrifugation as described in Methods, and fractions containing $[^{32}\text{P}]-\text{labeled RNA}$ greater than 0.25-0.3 kb in length was pooled. Size analysis of the pooled RNA probe indicated that its length was 0.3-0.5 kb (Fig. 29b).

The restriction endonuclease Kpn I has only one restriction site located 2.35 kb from the 5'-end of the cDNA template (Fig. 27). Therefore, Kpn I was chosen to digest the $[^{3}\text{H}]-\text{labeled cDNA\cdot plus DNA duplex}$. Digestion was performed as described previously. As a control, 1.65 µg of 5'-$[^{32}\text{P}]-\text{Hind III-digested \lambda phage DNA markers}$ were also digested with Kpn I in a parallel reaction in the presence of 5 µg calf thymus DNA. Analysis of the control digestion products by alkaline agarose gel electrophoresis revealed that in addition to the expected Hind III restriction fragments, an additional 4.6 kb fragment was detected which was the result of Kpn I digestion of the Hind III 23.1 kb fragment. The extent of Kpn I digestion of the $[^{3}\text{H}]-\text{labeled duplex DNA could not be estimated.}$

The Kpn I-digested cDNA\cdot plus DNA duplexes (14.3 ng) were analyzed by the technique of Southern blotting in conjunction with the $[^{32}\text{P}]-\text{labeled RNA probe}$ (Fig. 30). Prior to electrophoresis, 20 ng of $[^{3}\text{H}]-\text{cDNA}$ (940 cpm/pmole) estimated to be 2.5-4.0 kb in length was added to the digested duplex DNA to serve as an internal, LTR-specific reference. As additional controls, 115 ng of LTR-containing cDNA less than 3.0 kb in length that had been synthesized in the phosphate-buffered reconstructed reaction, and 1.4 ng of undigested $[^{3}\text{H}]-\text{labeled cDNA\cdot plus DNA duplexes}$ (5.0-8.0 kb) were fractionated in separate lanes. Analysis of the resulting autoradiogram revealed several interesting features (Fig. 30). There is evidence for the transfer and subsequent detection of both the digested
Fig. 30. Detection of LTR-Containing cDNA Transcripts Synthesized in a Phosphate-Buffered Reconstructed Reaction by Southern Blotting in Conjunction With Restriction Digestion by Kpn I. Genomic-length cDNA that had been converted into duplex DNA (mass ratio 0.55) and digested overnight with 16 units of Kpn I, was fractionated on an alkaline 1.2% (w/v) agarose gel at 40 volts (constant voltage) for 17 hr. The DNA-containing gel was blotted to DBM-paper for 24 hr as described in Methods, and hybridized with 5'-[\textsuperscript{32}P]-labeled sub 8-10S poly(A\textsuperscript{+}) RNA for 18 hr. The resulting blot was dried, and subjected to autoradiography as described in Methods. Lengths (in kb) refer to the relative positions of 5'-[\textsuperscript{32}P]-Hind III-digested \(\lambda\) phage DNA markers.

Lane 1 - 115 ng of control LTR-containing cDNA, 3.0 kb and less in length, and 5 \(\mu\)g calf thymus DNA.
Lane 2 - 1.4 ng of undigested [\textsuperscript{3}H]-cDNA-plus DNA duplexes (5.0-8.0 kb), 5 \(\mu\)g calf thymus DNA, and 15 \(\mu\)g calf thymus DNA primers.
Lane 3 - 14.3 ng of Kpn I-digested [\textsuperscript{3}H]-cDNA-plus DNA duplexes, 20 ng internal reference LTR-containing cDNA (1.8-4.0 kb), 5 \(\mu\)g Kpn I-digested calf thymus DNA, and 15 \(\mu\)g calf thymus DNA primers.
[3H]-labeled cDNA-plus DNA duplexes and the internal reference cDNA (lane 3). DNA corresponding to the original size of the duplex DNA (A) as well as minor amounts of the 2.35 kb Kpn I restriction fragment (B) is detectable. There was no specific DNA fragment in the 5.5 kb range which would indicate the presence of the second LTR. This interpretation is due in part to the detection of the heterogenously-sized duplex DNA (5.0-8.0 kb) in this region which contains the first copy of the LTR. If genomic-length DNA containing the second LTR is actually present, then it is indistinguishable from those transcripts containing the first LTR. The internal reference DNA was also detected. The size range was more heterogenous than expected, approximately 1.8-4.0 kb. Therefore, the 2.35 kb Kpn I restriction fragment is partially overlapped by the internal reference cDNA. The undigested control duplex DNA (lane 2) is barely detectable. This was due in part to the low level of material present. In contrast, the LTR-containing cDNA less than 3.0 kb in length was easily detectable (lane 1). No other DNA species were detected with the sub 8-10S RNA probe. The failure of the probe to detect either the calf thymus DNA carrier used in restriction digestion, or the DNA primers used in second strand synthesis, demonstrated the probes specificity although these DNA samples were present in all three lanes in μg quantities.

The transfer step was not entirely efficient, as evidenced by the behavior of the 5'-[32P]-Hind III-digested λ phage DNA markers. A successive decrease in the efficiency of transfer of the DNA markers was observed as the lengths of the marker fragments increased. As a result, the 4.4 kb and 6.6 kb fragments appeared to decrease in intensity, and the 9.4 kb fragment was barely discernible. Whether this phenomena
was due to inefficiency of transfer of large molecular weight DNA or to irregularities in the blotting procedure is not known. It is apparent, however, that the amount of detectable duplex DNA greater than 5.0 kb transferred to the blot was much less than the amount of the 2.35 kb \textit{Kpn I} fragment (lane 3), even if allowance is made for the amount of internal reference cDNA present in lane 3. Although LTR-containing cDNA in the 5.0-6.0 kb range was detected by the labeled RNA probe, it cannot be concluded whether these transcripts contained the second LTR. The uncertainties in digestion by \textit{Kpn I} suggest that much of these species could represent undigested cDNA greater than 5.0 kb in length which contain only the first copy of the LTR.
CHAPTER 4

DISCUSSION

The reconstructed reaction was used as a vehicle to further document the process of reverse transcription. An earlier study of the AMV reconstructed reaction (Olsen, 1982) focused on the biochemistry of reverse transcription at the 3'-end of the 35S RNA genome. While some of the data from the present study confirms some of these findings, the major intent in these studies was to extend the previous work. A new buffering system for more efficient reverse transcription is described, including an investigation of its effects on the cDNA synthesis following the first transcriptional jump. Comparison with the Tris-buffered system demonstrated that the phosphate-buffered system can support synthesis of identical DNA species of both minus and plus polarity, and that genomic-length cDNA is synthesized at a faster rate and in larger quantities. The cDNA transcripts synthesized in the phosphate buffered reconstructed reaction were analyzed by restriction mapping using a direct approach based upon the various lengths of the cDNA transcripts. This method of mapping could be extended to generate specific cDNA probes designed to investigate the mechanism of plus DNA synthesis. Furthermore, the results of this study suggest that the basic machinery for reverse transcription is present within the reconstructed system, and that purified reverse transcriptase is capable of synthesis of DNA indistinguishable from those species found early in infected cells. Use of a reconstructed system to study retrovirus replication is useful in that the effects on
reverse transcription due to a single component or factor can be examined. A logical extension of these studies would be investigation of purified, ancillary factors on the process of reverse transcription which are believed to play an as yet undefined role in retroviral replication.

Optimization of the Reconstructed Reaction in Phosphate Buffer

A key criteria for characterization of cDNA synthesis was synthesis of genomic-length cDNA. To achieve this, a new buffer system was investigated and reaction components were varied to determine the conditions established for optimum synthesis of genomic-length cDNA.

Potassium Phosphate as Buffer - A major discovery was that under certain conditions, potassium phosphate (pH 8.0) appears to be a better buffer for DNA synthesis than Tris-HCl. Potassium phosphate was tested as a buffer since our stocks of reverse transcriptase were stably stored in 150 mM potassium phosphate (pH 8.0). In comparison with the standard buffer routinely employed in reverse transcription, 50 mM Tris-HCl (pH 8.1), reactions incubated in 50 mM potassium phosphate (pH 8.0) resulted in greater amounts of genomic-length DNA (Fig. 12), a faster rate of DNA synthesis (Fig. 6), higher yields of small-sized DNA, much of which is of plus polarity (Fig. 15 and 16), and reduced levels of hairpin DNA (Fig. 17 and 18). The presence of 50 mM potassium phosphate (pH 8.0) may aid in elongation of cDNA following the first transcriptional jump, as after 2 hours of incubation, only minor amounts of cDNA\textsuperscript{100} were detectable (Fig. 18). The net increase of DNA synthesis after 2 hours of incubation varied on the average from 10% to 25% greater than that observed for the Tris-buffered system.

Just why potassium phosphate was a better buffer than Tris-HCl is
not known. Reverse transcriptase is relatively stable for years when stored at -20 °C in 50% (v/v) glycerol, 10 mM DTT, and 150 mM potassium phosphate (pH 8.0). This is not the case when Tris-HCl is substituted for potassium phosphate. The presence of potassium phosphate may help to stabilize the reverse transcriptase by inhibition of phosphatases which could dephosphorylate and inactivate the enzyme (Tsiapalis et al., 1976, unpublished results of Wolkowicz, 1984). Indeed, purified reverse transcriptase, either in the phosphorylated or dephosphorylated form, when stored in the absence of potassium phosphate at -20 °C, loses activity at a fairly rapid rate (Tsiapalis et al., 1976; unpublished results). Phosphate has reportedly been used as a buffer in the oligo(dT)-primed reaction with less success than with Tris-HCl (Retzel et al., 1980). Furthermore, it was also reported that at physiological concentrations of phosphate ion, elongation of cDNA was inhibited. Although a similar inhibition of genomic DNA synthesis was seen when the phosphate concentration was increased to 78 mM (Fig. 9), this report is difficult to analyze since no details of reaction conditions were given, such as the type of phosphate salt, pH, or concentration. However, optimal conditions for the tRNA<sup>Trp</sup>-primed reaction might very well differ from the oligo(dT)-primed reaction because of the requirement for a transcriptional jump in the former. Nevertheless, potassium phosphate may be a viable alternative as a buffer for RNA-directed DNA synthesis.

**Effect of 35S RNA Concentration**

Early studies of reconstructed reactions used high concentrations of 35S RNA template (greater than 200 µg/ml) and oligo(dT) primers for synthesis of genomic DNA (Kacian and Myers, 1976; Myers et al., 1977). LA-
ter studies of reconstructed reactions utilizing either the oligo(dT)-
primed reaction (Retzel et al., 1980) or the tRNA$^{\text{Trp}}$-primed reconstructed
reaction (Schulz et al., 1981, Olsen, 1982) employed lower concentrations
of AMV 35S RNA (4 µg/ml, 10 µg/ml, and 25 µg/ml, respectively). The
finding that a 35S RNA concentration of 20 µg/ml (Table 1, Fig. 8b) re-
sulted in relatively high yields of genomic DNA agrees closely with the
last two studies. Although lower concentrations of 35S RNA resulted in a
higher percentage of template transcribed (0.07 ng DNA/ng RNA), the total
amount of DNA synthesized was not sufficient for further experimentation.
Varying the RNA concentration from 5-20 µg/ml did not significantly change
the size distribution of the DNA synthesized. The finding that high RNA
concentrations of 41 µg/ml resulted in both a decrease in the total yield
of DNA as well as the expected decrease in efficiency may be best ex-
plained by the limiting amount of reverse transcriptase in the reaction
mixture. Therefore, the concentration of 35S RNA in the phosphate-buf-
fered reconstructed reaction was maintained at 20 µg/ml to ensure a favor-
able enzyme: template ratio.

**Effect of Cations**

Not surprisingly, the concentration of cations in the reconstructed
reaction influenced both the size of the DNA transcripts as well as the
total amount of DNA synthesized. The optimal amount of MgCl$_2$ for genomic
length DNA synthesis was found to be 5-6 mM at a concentration of 0.63
mM 4 dNTP (Fig. 8b). This concentration also resulted in the highest
yield of DNA (Table 2). Similar results were reported by Olsen (1982)
in the Tris-buffered reconstructed reaction, but in contrast to another
study (Schulz et al., 1981) which utilized a MgCl$_2$ concentration of 10
mM. Studies of the AMV 35S RNA·oligo(dT)-primed reaction reported that a concentration of 3 mM MgCl₂ resulted in synthesis of large DNA transcripts (Myers et al., 1977). Concentrations greater or less than 5-6 mM of MgCl₂ in the phosphate-buffered reconstructed reaction clearly inhibited genomic DNA synthesis (Fig. 8). The optimum MgCl₂ concentration may be affected by the concentration of the deoxynucleoside triphosphates (dNTPs) in the reaction mixtures. This suggestion is supported by the high concentration of each dNTP (0.5 mM) used when a correspondingly high concentration of MgCl₂ (10 mM) was employed (Schulz et al., 1981). Comparison with the work of Olsen (1982) indicates that the phosphate-buffered system is more sensitive to the effects of MgCl₂ than is the Tris-HCl system. This phenomena may be due in part to the formation of insoluble magnesium-phosphate complexes which could remove Mg²⁺ from the solution. In support of this contention, extremely low levels of DNA greater than 3.0 kb were synthesized in a phosphate-buffered reconstructed reaction containing 12 mM MgCl₂ (Fig. 8d). Furthermore, it was noted that addition of MgCl₂ directly to undiluted phosphate buffer resulted in the immediate formation of a white crystalline precipitate. This could be avoided by adding the MgCl₂ last to the reaction mixture, when the potassium phosphate buffer has been diluted.

The finding that 150 mM Na⁺-K⁺ resulted in maximal amounts of genomic DNA in the phosphate-buffered reaction (Fig. 10) is identical to previous studies utilizing either the Tris-buffered tRNA⁰⁻Prp-primed (Olsen, 1982) or the oligo(dT)-primed (Retzel et al., 1980) reconstructed reactions. This concentration also resulted in the highest yield of DNA, which is in contrast to the work of Olsen (1982) who reported that con-
centrations of 74-100 mM Na\(^+\)-K\(^+\) gave the greatest amount of DNA, albeit
of subgenomic length. The phosphate-buffered reaction was not as sensi-
tive to minor fluctuations in monovalent cation concentration as was the
Tris-buffered reaction (Retzel et al., 1980; Olsen, 1982). It is of in-
terest to note that the physiological concentration of Na\(^+\)-K\(^+\) is approx-
imately 150 mM.

Effects of Temperature and Substrate Radioactivity on the Phosphate-
Buffered Reconstructed Reaction

Other factors such as deoxyribonucleoside triphosphate (dNTP), con-
centration, temperature, and specific activity of the radio-labeled dNTP
most likely also play a role in efficient reverse transcription in a re-
constructed system.

The optimum temperature for incubation of the reconstructed reaction
was 39 °C (Table 4). Incubation at 37 °C resulted in a 8% decrease in
DNA synthesis, similar to the 2% decrease observed by Olsen (1982). At
temperatures lower than 37 °C, the decrease in DNA synthesis was more
dramatic. At 34 °C, the rate fell to 70% of the amount observed at 39 °C.
Similarly, increasing the temperature of incubation beyond 41 °C also de-
creased the yield of DNA. These results agree with other studies involv-
ing the AMV tRNA \text{Trp}-primed or oligo(dT)-primed reaction (Retzel et al.,
1980; Olsen, 1982).

Varying the concentration of labeled dCTP from 0.03 mM to 0.1 mM
also had little effect on the size of the DNA transcripts or the overall
yield, which is in agreement with previous work (Olsen, 1982). Decreasing
the concentration of dCTP to 7.5 \(\mu\)M inhibited the synthesis of genomic
DNA, an effect which has been reported previously in the AMV oligo(dT)-
primed reaction (Collett and Faras, 1977; Retzel et al., 1980). The latter study also reported that concentrations of the four dNTPs of 1 mM each also inhibited genomic DNA synthesis in an oligo(dT)-primed reaction. However, in the present study, as well as an earlier study (Olsen, 1982), no such inhibition was observed in the tRNA_{Trp}-primed reconstructed reaction.

The primary effect of high specific activity (2000-4000 mCi/mmol) of $\alpha$-$[^{32}P]$-dCTP was a decrease in the total amount of DNA synthesis (Table 5). No significant change in the distribution of the DNA population was apparent when freshly prepared $\alpha$-$[^{32}P]$-dCTP was used (Fig. 11). Since $[^{32}P]$ is a strong beta emitter, the resultant energy due to radioactive decay is quite intense, and might directly affect the reverse transcriptase by an unknown mechanism. The ability of the enzyme to elongate cDNA in the presence of $\alpha$-$[^{32}P]$-dCTP of high specific activity did not seem to be impaired. The observed decrease in DNA synthesis was analogous to use of a more dilute concentration of reverse transcriptase. The enzyme did not appear to be irreversibly damaged or modified since a decrease in the percent of genomic DNA might be expected if this was the case. Reactions incubated in the presence of high specific activity $[^{3}H]$-dCTP did not show a similar effect, and no decrease in DNA synthesis was noted (Fig. 11). The possibility that the $\alpha$-$[^{32}P]$-dCTP preparations contained contaminants inhibitory to reverse transcription cannot be completely ruled out, although when the specific activity was decreased to 1000 mCi/mmol (2000 cpm/pmol) no decrease in DNA synthesis was evident. Although not performed, detection of contaminants could be done via high pressure liquid chromatography (HPLC) with an anion-exchange micropar-
ticate silica matrix, or even by thin layer chromatography.

Genomic DNA synthesis as well as the total yield of DNA was inhibited by use of $\alpha^{[32P]}$ preparations that had undergone more than one half-life of decay (Table 5, Fig. 11). Under normal storage conditions (-20 °C in 50% (v/v) ethanol), radiochemical impurities may be generated at the rate of 5% per week. The half-life of $[32P]$ is short ($t_{1/2}=13.5$ days) and the decayed product resembles dCTP except that the phosphate in the $\alpha$-position is modified to sulfate. This dCTP analogue could inhibit RNA-directed DNA synthesis in several ways. First, the dCTP analogue could bind to the catalytic site, possibly in a reversible manner. Second, the dCTP analogue could be inserted in the growing strand of DNA. If true, then the inhibition of genomic DNA synthesis could be a result of the difficulty of the reverse transcriptase to either cleave the dCTP analogue between the $\alpha$ and $\beta$ positions, or to ligate the dCMP analogue to the nascent DNA chain. Whatever the mechanism, it is evident that freshly prepared $\alpha-[32P]$-dNTP should be used for maximum synthesis of genomic DNA in the phosphate-buffered reconstructed reaction.

**Rate of DNA Synthesis**

The rate of DNA synthesis in the phosphate-buffered system was greater than in the Tris-buffered system (Fig. 6). Genome-length DNA was present less than one hour after initiation of DNA synthesis (Fig. 7). Assuming genomic DNA to be approximately 7.8-8.0 kb in length, the rate of DNA synthesis in the phosphate-buffered system is on the average 130-140 nucleotides min$^{-1}$ at 39 °C. This is somewhat faster than reported for the Tris-buffered system (120 nucleotides min$^{-1}$) as reported by Olsen (1982) and as found in the present study (120-125 nucleotides min$^{-1}$). This rate
is also faster than the 30 nucleotides·min\(^{-1}\) observed upon infection of quail cells by avian sarcoma virus (Varmus et al., 1978) but only 50% of the rate found in the RAV-2 endogenous reaction (Boone and Skalka, 1981).

The kinetics of DNA synthesis in the phosphate-buffered reconstructed reaction are biphasic, with an initial rapid phase of DNA synthesis followed by a slow phase of several hours (Fig. 6). The slow phase is marked by the accumulation of genomic-length DNA transcripts (Fig. 7). Similar observations were noted in an earlier study of the Tris-buffered reconstructed reaction (Olsen, 1982), in which the slow phase continued for 20 hours. Biphasic kinetics have been reported by other researchers as well (Fuadinaga et al., 1970; Quintrell et al., 1971; Boone and Skalka; 1981).

**Detection of Plus DNA Synthesis in the Phosphate-Buffered Reconstructed Reaction**

Evidence of plus DNA synthesis was obtained during the current studies involving the phosphate-buffered reconstructed reaction. Oligo(dT)-primed reactions incubated in the presence of 4 mM sodium pyrophosphate (Fig. 14b) were not capable of synthesis of the small-sized DNA species seen previously (Fig. 8), although this class of DNA was present in reactions incubated in the absence of sodium pyrophosphate (Fig.14a). Sodium pyrophosphate is a reported inhibitor of RNase H activity and second strand DNA synthesis (Myers and Spiegelman, 1978), which suggests that much of the small-sized DNA population synthesized in the tRNA\(^{Trp}\)-primed reaction may be of plus polarity. Hybridization to AMV 35S RNA template of DNA 0.2-2.5 kb that had been synthesized in a phosphate buffered reconstructed reaction indicated that 40% of the DNA products did
not hybridize (Fig. 15). Hybridization experiments involving the total DNA products synthesized in a phosphate-buffered reconstructed reaction revealed that most of the unhybridized DNA was less than 1.5 kb in length (Fig. 16). Fractionation of the small-sized DNA population by electrophoresis on 4.8% (w/v) polyacrylamide-7 M urea gels (Fig. 18) detected several major species of DNA. Of these species, three were detectable by Southern blotting with cDNA3 probe; plus DNA400, plus DNA300, and hpDNA (170 nucleotides). These species are anticomplementary to the 3'-end of the 35S RNA (3' LTR) and have been detected previously in the Tris-buffered reconstructed reaction with cDNA100 probes (Olsen and Watson, 1982). Additional plus DNA species up to 2.0 kb in length were detected with the non-specific cDNArep probe (Fig. 17). Similar species were also detected in the Tris-buffered reaction, although they did not exceed 1.5 kb in length (Olsen, 1982). These results strongly suggest that plus DNA synthesis is discontinuous. If it were continuous, then the cDNA3, and cDNArep probes would have detected identical plus DNA species.

Previous studies of the AMV reconstructed reaction (Olsen, 1982; Olsen and Watson, 1982) have shown that plus DNA400 and plus DNA300 are related to each other. Plus DNA400 contains an internal sequence that is deleted in plus DNA300, and the detection of both species by the LTR-specific probe suggests that they are located within the AMV LTR. Restriction mapping studies of the LTR (Olsen, 1982) suggested that these two plus DNA species are initiated and terminated at the same locations on the cDNA template. The mechanism by which this occurs is unknown, but may involve heterogeneity in the 35S RNA, such as deleted sequences,
or deletions may occur during the process of reverse transcription of the viral 35S RNA or cDNA template. Plus DNA\(_{300}\) may be an artifact of the reconstructed reaction, as this species was only slightly detectable in the AMV endogenous reaction (Olsen, 1982). Modification of the cation concentrations in the endogenous reaction reportedly decrease the amount of plus DNA\(_{300}\).

Hairpin DNA did not appear as a major product of the phosphate-buffered reconstructed reaction (Fig. 10) although it comprised 50% of the DNA species less than 0.2 kb (Fig. 18). Synthesis of hp DNA is a result of incorrect elongation of cDNA\(_{100}\) which does not undergo the correct transcriptional jump, and copies itself (Olsen, 1982). Hp DNA reanneals quickly to itself in hybridization experiments but can be detected by cDNA probes.

Hp DNA has been reported to be present in smaller amounts in the AMV endogenous reaction (Olsen, 1982). Similar findings were also reported for the avian sarcoma virus reconstructed and endogenous reactions (Swanson et al., 1981a). Hp DNA, like plus DNA\(_{300}\), may be an artifact of the reconstructed reaction as it represents only a small fraction of the AMV RNA genome. Although it has been suggested that hp DNA is involved in initiation of plus DNA synthesis (Collett and Faras, 1978), this would tend to preclude synthesis of the LTR in linear proviral DNA. However, hp DNA may play an unknown role in retrovirus replication.

Restriction Mapping of the AMV Complex cDNA Transcripts

Early attempts to construct a restriction map of the AMV complex with cDNA-RNA hybrids (Malloy and Symons, 1980) were unsuccessful. Several problems forced a different approach involving the use of cDNA-plus DNA du-
plexes as substrates for restriction endonuclease digestion. With the exception of Hind III, none of the restriction enzymes used in the current study were able to digest cDNA-RNA hybrids. Hind III digestion was non-specific, and yielded a heterogenous range of digested fragments which contained no distinguishable species. Upon further investigation, the Hind III preparation was found to be contaminated by nucleases. The addition of calf thymus DNA to act as a carrier did not result in digestion of the hybrids although an excess of the appropriate restriction endonuclease was present in the reaction. Of the potential candidates for restriction mapping of the cDNA template, only Eco RI was reported to be able to digest DNA-RNA hybrids. Other restriction endonucleases such as Kpn I, Bam HI, Xho I, and Hind III, had not been demonstrated to be able to digest RNA-DNA hybrids (Malloy and Symons, 1980). Hybridization of the cDNA transcripts to 35S RNA may have been incomplete in some instances. However, in several experiments hybridization was essentially complete yet subsequent analysis of the cDNA transcripts revealed no detectable digestion products. As a control, the restriction enzymes were able to digest double-stranded DNA, precluding the possibility that the restriction endonucleases were inactive. A final consideration in the decision to switch to cDNA-plus DNA duplexes was the large amounts of suitable 35S RNA required for hybridization. Therefore, alternatively, the second strand of DNA was synthesized with calf thymus DNA primers and presized cDNA transcripts (Summers, 1975; Summers et al., 1975; Taylor et al., 1978). Reverse transcriptase was substituted for the Klenow fragment of DNA pol I since it was readily available.

Synthesis of the second strand of DNA was dependent on the concen-
tration of the reverse transcriptase pool used. The extent of digestion of the DNA duplexes was dependent on the amount of plus DNA synthesized. In general, mass ratios of plus DNA/cDNA less than 0.5 were insufficient for mapping purposes although in several experiments mass ratios of 0.4 and less were sufficient to generate detectable restriction fragments.

A consistent side reaction noted was the apparent ability of the cDNA template to self-prime itself in the absence of exogenous primers (Fig. 19). This phenomena has been noted by other investigators (Schulz et al., 1981). Although not rigorously proven, it is believed that at the termination of cDNA synthesis by reverse transcriptase, the cDNA template forms a small hairpin at the 3'-end of the nascent cDNA transcript, resulting in a free 3'-OH group which can act as a primer. No evidence has been presented thus far that suggests that reverse transcriptase can initiate DNA synthesis in the absence of a free 3'-OH group. Experimental conditions rule out the possibility of residual RNA fragments present which could act as primers. Even with the addition of 350 μg/ml calf thymus DNA primers, significant amounts of plus DNA (up to 29% of the total amount) were synthesized via cDNA template extension (Fig. 19). This side reaction could be minimized to less than 10% of the total amount of plus DNA synthesized by hybridization of the primers and template following heating and quick cooling of the mixture. Extension of the cDNA template was undesirable for two reasons. First, the presence of covalently linked [³H]-plus DNA and [³²P]-cDNA could result in generation of spurious restriction fragments. Second, extension of the cDNA template might not be sufficient to convert single-stranded restriction sites near the 5'-end of the cDNA transcript into double-stran-
Previous restriction mapping studies of the MAV-1, MAV-2, or AMV genomes have utilized DNA intermediates isolated from infected cells (Bergmann et al., 1980, 1981; Baluda et al., 1983) or cDNA from the reconstructed reaction hybridized to plus DNA (Olsen, 1982; Olsen and Watson, 1982). Due to these efforts, a detailed restriction map was available for AMV and MAV-1, which provided a check on mapping results obtained using cDNA synthesized in the phosphate-buffered reconstructed reaction. One complication previously mentioned using this type of approach, is that several types of viral RNA species constitute the AMV complex. Except for the amv gene insert in AMV, the restriction maps are quite similar.

The mapping data obtained for the restriction enzymes Bam HI (Fig. 22) and Hind III (Fig. 24 and 26) resulted in detection of restriction sites on the cDNA template identical with the MAV-1 genome (Bergmann et al., 1980, 1981; Baluda et al., 1983). Within the size ranges of the cDNA transcripts used (Table 6), all the known MAV-1 restriction sites were detected. With the exception of the known Hind III restriction site in the 3' LTR, no other sites specific for the AMV genome were detected. Since MAV-1, MAV-2, and AMV share a common 3' LTR region, restriction sites specific for the 3' LTR of AMV are indistinguishable from those in MAV-1 and MAV-2. In contrast, the Xho I mapping results (Fig. 25) were more ambiguous. In addition to the expected restriction fragments generated, two additional fragments were detected, 2.1 kb and 1.7 kb, respectively. The 2.1 kb fragment could result from incomplete digestion of the DNA duplexes, or it could represent a unique restriction
fragment as yet unmapped. If the latter suggestion is true, then the restriction site would most likely be located on the MAV-2 genome, as it has not been extensively mapped (Bergmann et al., 1980). The MAV-2 genome might very well lack the 0.46 kb Xho I restriction site. A more likely explanation is that the 2.1 kb fragment is the result of incomplete digestion. Support for this contention arises from uncertainties in the extent of conversion of the Xho I restriction site into dsDNA. The amount of plus DNA synthesized was 70-85% of the amount of available template. This suggestion could be refuted by digestion of cDNA-plus DNA duplexes that were completely double-stranded. Alternatively, isolation of separate, purified lots of AMV, MAV-1, and MAV-2 RNA, followed by mapping of their respective cDNA transcripts, would provide a definitive answer. Similarly, the minor 1.7 kb fragment did not fit the available restriction map. Unlike the 2.1 kb fragment, it is not the summation of two or more Xho I restriction fragments and possibly represents a new, as yet unknown, restriction site. If this suggestion is true, it is probably located on the MAV-2 or AMV genome as this fragment was detected in minor amounts. Further experimentation is necessary to resolve this question.

The presence of more than one type of RNA species in the AMV complex was demonstrated in mapping experiments involving Eco RI (Fig. 21, 26). The possibility that Eco RI may be cleaving sequences closely related to the recognition sequence is not likely. Experiments in which one strand of dsDNA was modified at the Eco RI recognition site resulted in only the unmodified strand undergoing cleavage (Bishop, 1979) by Eco RI. Other reports have reported the ability of Eco RI to bind to nonspec-
ific sites on a variety of polynucleotides (Goppelt et al., 1980; Langowski et al., 1980) although nonspecific cleavage was not detected. If the two additional restriction fragments are from AMV, then our standard RNA preparations contain this RNA specie in significant amounts. The mapping results obtained with Kpn I did not clarify this question. Any expected restriction fragments from AMV would be obscured by the overlap of secondary restriction fragments generated by the MAV-1 cDNA transcripts (Fig. 20, 26). Resolution of this question in future experiments could include the use of restriction enzymes specific for AMV, such as Sma I, Bal I, and Sal I (Fig. 4). Detection of any restriction fragments generated with these enzymes would suggest that AMV is present in detectable quantities, and an estimation of the percent of AMV RNA in the AMV complex could be made.

Hgi A1 has not been used previously to map either the AMV, MAV-1, or MAV-2 genomes. Therefore, the data for two Hgi A1 restriction sites is presented for the first time. The 0.51 kb fragment is located at the 3' LTR. Therefore, the AMV, MAV-1, and MAV-2 genomes should all contain this restriction site. The 2.6 kb fragment cannot be unequivably assigned to the MAV-1 genome, although the presence of this fragment in large quantities suggests that it is (Fig. 23). MAV-1 is believed to comprise the majority of the RNA species in the AMV complex (Bergmann et al., 1980, 1981). The 3.2 kb fragment may be the result of incomplete digestion by Hgi A1. In support of this, the extent of plus DNA synthesis was only 85% of the available cDNA. Another likely explanation is that one of the RNA species present in the AMV complex lacks a Hgi A1 restriction site. At the present, the data does not indicate which sug-
gestion is correct. Resolution of this question could come from mapping experiments involving separate lots of MAV-1, MAV-2, and AMV RNA as mentioned for Xho I.

Some minor problems occurred during mapping which could be circumvented in future experiments. Mapping of the 3'-end of the cDNA genome (5' LTR) was handicapped by insufficient genomic DNA. This could be overcome in two ways. First, the reconstructed reaction could be further optimized with respect to genomic DNA synthesis. This would entail use of additional components not available at the present time, such as addition of viral-associated proteins to the reconstructed reaction. One candidate, the viral-associated protein pl2, is thought to be associated with the 35S RNA genome in the native virion and may act to relax the RNA (Leis et al., 1983). A more practical solution would be to scale up the reaction. This suggestion would require additional amounts of purified reverse transcriptase and viral 35S RNA. Another problem of a technical nature involved separation of the cDNA transcripts by sedimentation velocity centrifugation. On several occasions, considerable heterogeneity in the sizes of the cDNA transcripts was evident (Fig. 26). This resulted in difficulties in identification of certain restriction fragments due to the background of heterogenous cDNA (Fig. 26). To avoid this, fractionation of the cDNA transcripts on low-melting agarose gels would allow for cDNA transcripts of narrower size ranges to be selected. One drawback to this method is that increased amounts of cDNA transcripts would be necessary to ensure that sufficient templates are available for plus DNA synthesis. Since the extent of restriction digestion of duplex DNA was directly proportional to the amount of plus DNA synthesized,
the key step in successful mapping was synthesis of plus DNA in high yields. Use of the Klenow fragment of DNA pol I could be an alternate choice for second strand synthesis (Taylor et al., 1978). Other researchers have utilized highly concentrated reverse transcriptase for second strand synthesis with corresponding high yields (Schulz et al., 1981). These minor problems were not insurmountable, as evidenced by the successful detection of restriction sites for six different restriction endonucleases.

Although this method of mapping was developed for analysis of the restriction sites located on the cDNA template synthesized in the phosphate-buffered reconstructed reaction, it can be applied to other systems other than AMV. Theoretically, any viral or non-viral RNA can be mapped by this method, provided that synthesis of cDNA is initiated at a specific, unique location. Poly(A)-containing RNA could be primed with a specific DNA or RNA containing an oligo(dT) or poly(rU) tail ligated to a mono-, di-, or even trinucleotide complementary to the 3'-end of the RNA. Furthermore, this direct approach does not rely upon more complicated mapping procedures such as double digestion, partial digestion, and other methods which have made restriction mapping laborious at times (Smith and Birnstiel, 1976). Mapping of RNA species using cDNA transcripts synthesized with reverse transcriptase constitutes an alternative approach to traditional mapping procedures, and has been used by other researchers (Taylor et al., 1978).

Detection of the Second Long Terminal Redundancy (LTR)

The DNA products synthesized in the Moloney murine leukemia and murine sarcoma virus endogenous reactions have been shown to contain
both the 3' and 5' LTRs (Benz and Dina, 1979; Gilboa et al., 1979; Dina and Benz, 1980). No evidence has been presented thus far which demonstrates the capability of the reconstructed reaction to synthesize genomic DNA containing the second (5' LTR). An attempt was made in the present study to try to determine whether the genomic cDNA transcripts synthesized in the AMV phosphate-buffered reconstructed reaction contained the second LTR by Southern blotting (Southern, 1975).

Two possible probes were considered for use to detect the second LTR; plus DNA\textsubscript{400} and sub 8-10S poly(A)\textsuperscript{+} RNA (Shank et al., 1978a). Plus DNA\textsubscript{400} was not used because it was not available in adequate quantities. The RNA probe was very selective for the LTR, as determined by its size (Fig. 29), and its specificity for cDNA complementary to the 3'-end of 35S RNA. The RNA probe did not detect the carrier calf thymus DNA, although it was present in all lanes of the blot in μg amounts (Fig. 30). The sensitivity of the probe was apparent in its ability to detect nanogram amounts of LTR-containing cDNA (Fig. 30). Since the 3' and 5' LTR are believed to contain identical primary sequences, a LTR-specific probe cannot distinguish between them. To overcome this obstacle, restriction digestion of genomic DNA that had been converted into duplex DNA was necessary to provide a means to distinguish between both LTRs. Restriction mapping in conjunction with Southern blotting has been used to successfully detect the second LTR in avian sarcoma proviral DNA isolated from infected cells (Hughes et al., 1978; Shank et al., 1978b). Analysis of the resulting blot (Fig. 30) did not provide a definitive answer to this question. If genomic cDNA transcripts synthesized in the phosphate-buffered reconstructed reaction do contain elements of the
second LTR, then the procedure was not sufficiently selective or sensitive enough to detect it. Since the extent of digestion of duplex DNA by restriction endonucleases is directly proportional to the amount of plus DNA synthesized (55% in this experiment), uncertainties remain as to the extent of conversion of the Kpn I restriction site into dsDNA.

To resolve this question experimentally, the following modifications would be helpful in future experiments using this approach. First, assuming that adequate quantities of genomic cDNA are available, only cDNA transcripts longer than 6.5 kb should be used as template for second strand synthesis. This pool of cDNA, even if partial digestion by Kpn I or another suitable restriction enzyme did result, could easily be distinguished from the 5.5 kb Kpn I restriction fragment. As a control, [32P]-labeled cDNA could be synthesized in a parallel reaction, and treated similarly to the sample cDNA. Second, synthesis of the second strand of DNA with both control and sample cDNA should be extensive (90% or greater) to ensure adequate coverage of the Kpn I restriction site. Use of the Klenow fragment of DNA pol I or high concentrations of purified AMV reverse transcriptase would be suitable for second strand synthesis. To aid in confirmation of the extent of plus DNA synthesis, an aliquot of the control duplex could be subjected to S1 nuclease digestion to estimate the percent of single-stranded cDNA remaining. Third, the extent of digestion by Kpn I could be determined by electrophoresis of the control duplex. In addition, digestion of intact, unlabeled lambda phage DNA by Kpn I followed by 5'-end labeling would serve as an additional control as the locations of the Kpn I restriction sites on lambda phage DNA are well known. Fourth, the efficiency of transfer of large DNA fragments
are not as efficient as the transfer of small size DNA fragments. The rate of transfer depends on the molecular weight of the DNA specie and the porosity of the gel. Transfer of large size DNA may be improved by stringent hydrolysis of the DNA-containing gel prior to transfer by de-purination with acid (Wahl et al., 1979), use of nitrocellulose filters in conjunction with lengthened blotting times, or use of a lower mass percent of agarose (Shank et al., 1978b). Extended periods of blotting to nitrocellulose filters (up to 60 hours) have been used by some researchers with good results (Shank et al., 1978b) whereas blotting periods greater than 24 hours with DBM paper may increase background or cause diffusion of the transferred DNA. Another possible method to ensure sufficient breakage of the DNA may be to stain the DNA-containing gel with ethidium bromide, followed by exposure to UV irradiation. Care must be taken not to hydrolyze the DNA too much, as DNA fragments less than 0.3 kb are too short to bind efficiently. Use of labeled DNA markers of known molecular weight and distribution would be ideal for preliminary studies to maximize transfer of large fragments of DNA. One alternative to this approach could be use of a restriction enzyme such as Bam HI. This enzyme is capable of digesting genomic cDNA into multiple restriction fragments, with the LTR-containing fragments relatively short in length (less than 3 kb) but distinguishable from each other (Shank et al., 1978a; Bergmann et al., 1981; Baluda et al., 1983). This would avoid extensive modification to the blotting procedure but would require additional mapping data. Finally, use of the LTR-specific sub 8-10S poly(A)$^+$ RNA probe could be used, as it has been shown to be sufficiently selective for cDNA containing a LTR (Fig. 30).
Although the presence of the second LTR in the genomic DNA transcripts synthesized in the phosphate-buffered reconstructed reaction cannot be confirmed, neither can the possibility that it is not present be excluded. The procedure used to attempt to detect the second LTR demonstrated that it may be possible to successfully answer this question. A similar approach, following the modifications as set forth, should be capable of providing a definitive answer to this question.

Summary

Conditions for and properties of reverse transcription were examined using the optimized AMV RNA-directed reconstructed reaction. The enhanced effects of potassium phosphate on RNA-directed DNA synthesis demonstrated its suitability as a buffer system. Compared to Tris-HCl, the DNA products of the phosphate-buffered system were synthesized in larger quantities at a faster rate, with a greater proportion of these transcripts at or near genome-length. Other species of DNA were detected which are similar to those synthesized in the Tris-buffered system (Olsen, 1982). Plus DNA synthesis was extensive, apparently discontinuous, and species up to 2.0 kb in length were detectable. Several discrete species of plus DNA were detected, including plus DNA_{400}. This latter species is believed to play a role in the second transcriptional jump during proviral DNA synthesis.

A restriction map of the AMV complex cDNA was compiled and was based upon cDNA transcripts synthesized in the phosphate-buffered system. Multiple restriction sites for six enzymes were located at or near the 5'-end of the cDNA template (3' LTR). With one exception, these sites were identical to those mapped for AMV, MAV-1, and MAV-2 DNA isolated from in-
fected cells (Baluda et al., 1983). Two new restriction sites were mapped in the AMV complex with Hgi Al, a restriction enzyme not previously used to map the AMV complex (Bergmann et al., 1982). Furthermore, the mapping results demonstrated that the cDNA transcripts synthesized in the phosphate-buffered reaction are biochemically similar to those found in infected cells. The mapping procedure was simple, direct, and could possibly be applied to other RNA species other than AMV. Specific cDNA probes could be generated from this mapping procedure to investigate further the mechanism of plus DNA synthesis.

An attempt to detect the second LTR in genomic DNA transcripts by Southern blotting in conjunction with restriction digestion using Kpn I was not successful. Although LTR-containing cDNA was detected, the procedure was not sufficiently selective to discriminate between the first and second LTRs. This question could not be resolved in this study due to lack of purified template 35S RNA. A more stringent approach based upon this method was outlined, which could perhaps answer this question.

Undoubtedly, the reconstructed reaction is lacking in certain, as yet unknown, components which are present in the endogenous reaction as well as in infected cells. However, since many of the DNA structures observed in vivo are generated in the reconstructed reaction, it would appear that this system is useful. The advantage of the reconstructed reaction over other approaches is that the effect(s) of a single component on RNA-directed DNA synthesis can be examined in depth, as demonstrated by the current study. Studies involving the retroviral reconstructed reaction could be extended by inclusion of other suspected factor(s) which might play a role in reverse transcription. Identification
of these components and their effect(s) on reverse transcription in a reconstructed system would enhance our knowledge of retroviral replication to a greater degree.
REFERENCES


