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Title: Vaccinia Virus Ribonucleotide Reductase: Gene Sequencing, Intracellular Localization, and Interaction with a DNA-binding Protein.

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Christopher K. Mathews, Ph.D.

Vaccinia virus infected monkey kidney cells had been previously shown to have an increased ribonucleoside diphosphate reductase (RR) activity. DNA from mutant virus resistant to hydroxyurea were digested with restriction endonucleases and were shown to have substoichiometric amounts of the *Hind* III F fragment. Additional information from Southern blotting experiments localized the putative small subunit (R2) gene to the left end of the *Hind* III F fragment of the vaccinia virus genome. The entire open reading frame of the R2 gene and the flanking regions was sequenced and the translated sequence found to be 80% homologous to the mouse R2 polypeptide.

A combination of *in situ* and *in vitro* experiments addressed the question of macromolecular interactions involving vaccinia ribonucleotide reductase (RR). Replication of double stranded viral DNA occurs in very discrete loci in infected cells and these DNA factories can be isolated from gently lysed cell in sucrose gradients. RR was detected at low levels (less than 5% of the total R2) with the rapidly sedimenting DNA by using antibodies against RR. *In situ* cross-linking experiments were attempted with no specific interaction determined at this time. Immunolocalization experiments have given evidence for localization of large subunit (R1) polypeptide to the viral inclusion bodies.

The most conclusive results utilized anti-idiotypic antibodies against the antibodies to R2 protein. Immunolocalization experiments have shown the putative R2 binding protein to be localized at the sites of viral DNA synthesis. Immunoprecipitations show a single predominant viral polypeptide which also has proven to be a DNA binding (phospho)protein. Screening a lambda phage expression library of vaccinia with the anti-idiotypic antibody localized the

binding site to the carboxy terminal 81 amino acids in open reading frame I-3 of the vaccinia genome. The open reading frame was cloned into a pET11c expression vector and the partially purified recombinant protein was shown to have specificity for single-stranded DNA as well as stimulate vaccinia RR activity.

Vaccinia Virus Ribonucleotide Reductase: Gene Sequencing, Intracellular Localization, and Interaction with a DNA-binding Protein.

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Chapter I
General Background

I.1 Introduction

Life can be viewed as a hierarchical organization of systems. At the molecular biology level gene regulation controls the amounts of different gene products depending upon the needs of the cell. At the biochemical level a great deal of attention has been focused on individual enzymes and small molecules which interact with them (substrates and effectors). Transient and/or weak interactions between macromolecules which help integrate metabolic processes are more poorly defined in many pathways. This thesis focuses on exploring protein-protein interactions which may modulate deoxyribonucleotide metabolism. A general inventory of this part of the biochemical machine has been established. What is not known with certainty is how these processes are integrated and connected. A summary of the metabolic pathway and allosteric regulation mechanisms (molecular level), ribonucleotide reductase (macromolecular), protein machines (inter-macromolecular), and vaccinia virus (biological system) lay the background for the thesis results which follow.

I.2 An Overview of Deoxyribonucleotide Metabolism

DNA replication can be considered as two distinct but interdependent processes: 1) the polymerization of building blocks into macromolecular products; and 2) the synthesis of those building blocks. The building blocks are synthesized from small molecules to form ribonucleotides, which are then reduced at the 2' position of the ribose moiety to yield the deoxyribonucleotide. The activated precursors for DNA polymerase are in the 5' triphosphate form and are incorporated as deoxyribonucleoside monophosphates (dNMPs), with a pyrophosphoryl group leaving in the process as the nucleotide is incorporated into DNA. Although much attention has been focused on the protein complexes involved in the polymerization of DNA, the mechanisms for coordinating and delivering the precursors to DNA polymerase remain less explored. A great deal remains to be answered as to the dynamics of the interactions among

enzymes involved in precursor metabolism and their connection to DNA synthesis. The necessity for fine coordination of DNA synthesis and precursor metabolism is apparent from the small pools of dNTPs available at any given time in the replication cycle. The DNA precursor pool sizes represent about one percent of a genome equivalent in *E. coli* and even less for mammalian cells. (Table I-1, Kornberg and Baker, 1992)

Table I-1 Deoxynucleotide levels in cells

DNA precursors	procaryotes (mM)	mammalian cells (mM)
dATP	0.18	0.013
dGTP	0.12	0.005
dCTP	0.07	0.022
dTTP	0.08	0.023

The pool of dNTPs is drawn from both *de novo* and salvage pathways in most cells (Figure I-1). The *de novo* pathways proceed via the synthesis of the ribonucleotides: ribose phosphate, specific amino acids, CO_2 and NH_3 are combined to form ribonucleoside monophosphates (rNMPs). The rNMPs are phosphorylated by specific kinases to the diphosphate level and reduced in most organisms by ribonucleotide reductase to form deoxyribonucleoside diphosphates (dNDPs). All dNDPs (and ribonucleoside diphosphates) are phosphorylated by a non-specific kinase, nucleoside diphosphokinase (NDPK), to deoxyribonucleoside triphosphates (dNTPs). In other organisms the reduction is done on ribonucleoside triphosphates (NTPs). The salvage pathway makes use of free bases and nucleosides released from nucleic acids which have been broken down by nucleases. At first glance this pathway appears to only supplement the *de novo* synthesis, but in some tissues and parasites it is the only means of dNTP generation and in general seems to help maintain a correct balance of deoxynucleotides for DNA synthesis (Reichard, 1988). With all four dNTPs needing to be produced in the correct ratios for delivery to the replication fork at rates proportionate to the nucleotide composition of DNA, a question arises as to how the organism balances the supplies. There are several points of regulation as studied in both the *E. coli*

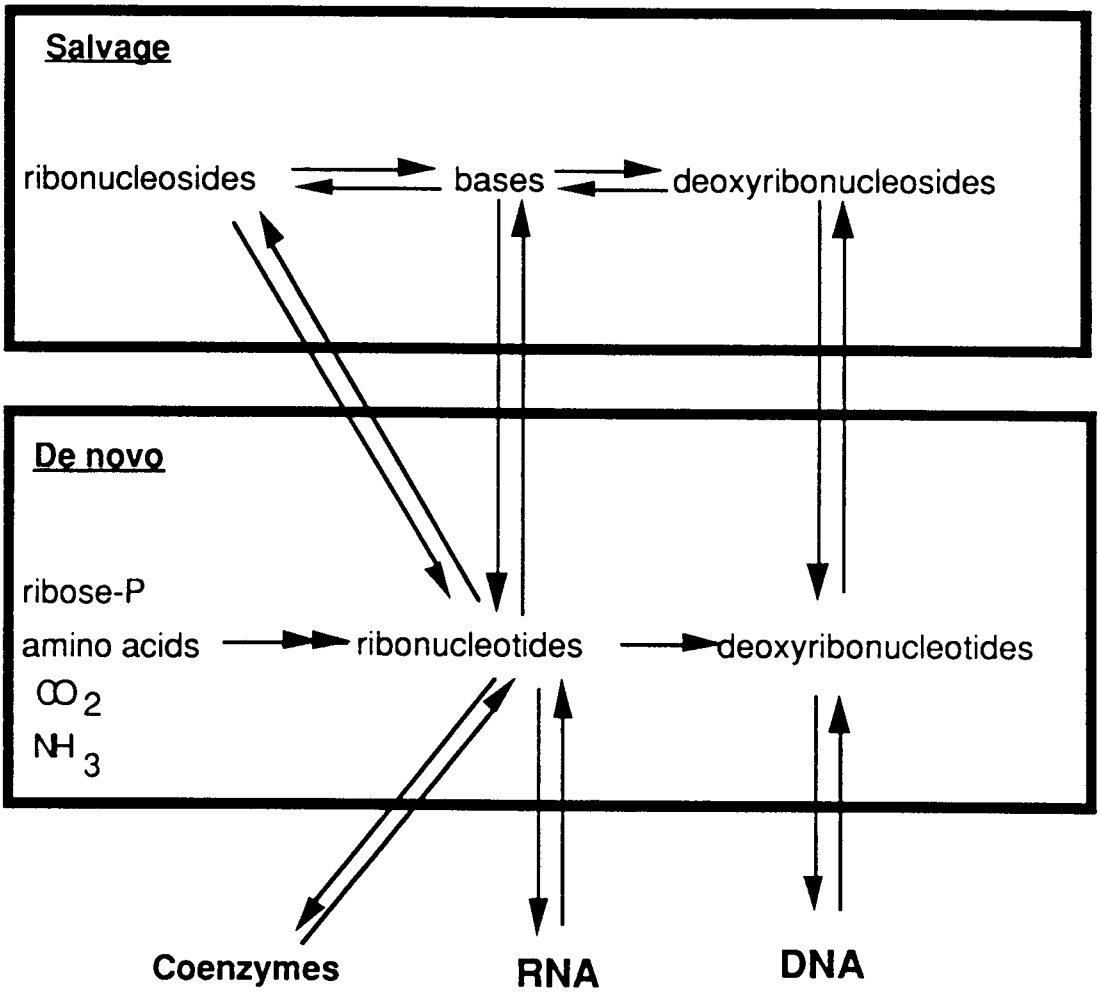


Figure 1-1 Overview of nucleotide metabolism

system and the mammalian system, which allow for the fine control of the ratios of the deoxynucleotides. For the *de novo* system the primary site of control resides with the reduction of ribonucleoside diphosphates to deoxynucleoside diphosphates (figure I-2). Ribonucleotide reductase (RR) can reduce all four common ribonucleotides, given the proper allosteric effector (dTTP, dGTP, dATP, ATP). Various feedback mechanisms involving product inhibition and substrate specificity allow for a primary level of control of the species for dTMP synthesis. A specific thymidylate kinase phosphorylates dTMP to dTDP from both the *de novo* and salvage pathways.

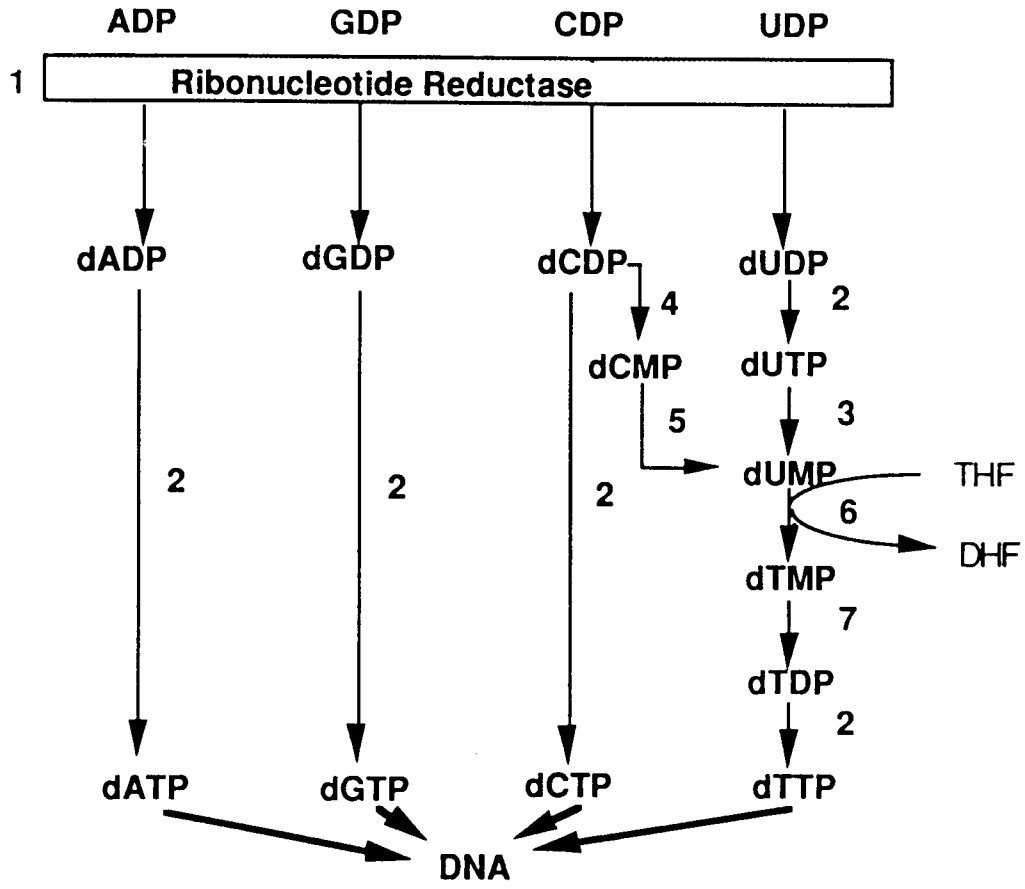


Figure 1-2 Denovo dNTP synthesis: The allosterically regulated enzymes are - ribonucleotide reductase (1) and dCMP deaminase (5). The other enzymes in the pathway are - nucleoside diphoshate kinase (2), dUTPase (3), (d)CMP kinase (4), dTMP synthase (6), and thymidylate kinase (7). THF = tetrahydrofolate; DHF = dihydrofolate.

The *de novo* synthesis system can be viewed as being supplemented and fine tuned by salvage, catabolism, and substrate cycling (Reichard, 1988). Studies on perturbations of dNTP pools have shown imbalances of dNTPs to cause a plethora of effects from single- and double-stranded DNA breaks (Ayusawa et al., 1986; Ingraham et al., 1986), to recombination (Kunz, 1982), mutagenesis (Nalbantoglu et al.,1987; Phear et al.,1987) and immune deficiency disease (Carrera et al., 1987). Changing the relative concentration of dNTPs in *in vitro* DNA replication affects fidelity (Kunkel et al., 1986) with the nucleotide in excess misincorporated at an increased rate. Substrate cycling (Figure I-3) would modulate the *de novo* pathway and help maintain the proper

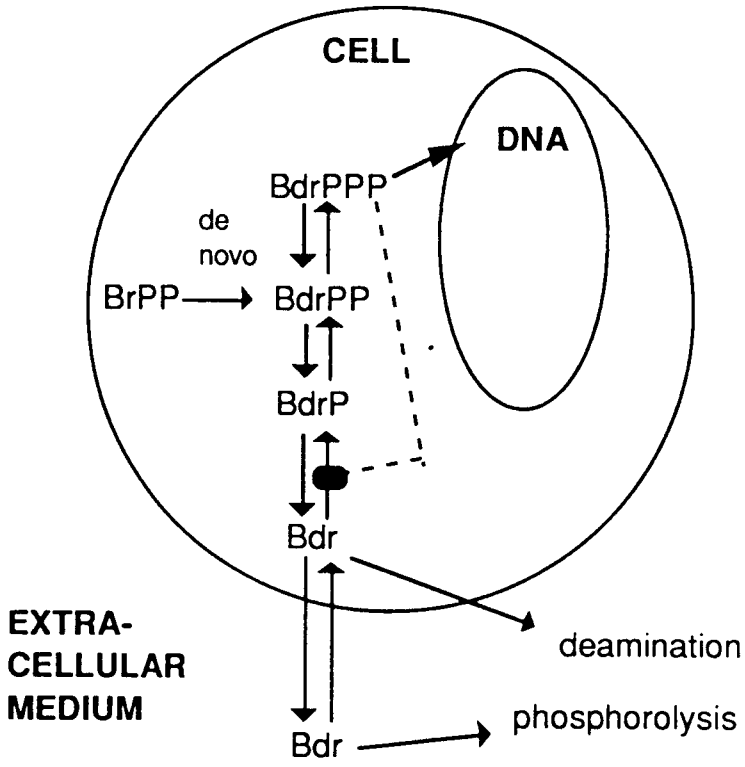


Figure I-3 Substrate cycling. The balance between the deoxyribonucleoside (Bdr) and the deoxyribonucleotides (BdrP, BdrPP, BdrPPP) can be shifted toward catabolism with the inhibition of a deoxyribonucleoside kinase by the dNTP. The intracellular concentration of deoxyribonucleosides is regulated by flux into and out of the cell.

balance of deoxyribonucleotides in order to minimize errors in DNA repair and replication.

Nucleosides pass through the cell membrane by facilitated diffusion, which involves a broad specificity permease (Plagemann and Wohlhueter, 1980). Most cells phosphorylate some deoxyribonucleosides, with mammalian cells containing kinase activities for all four deoxyribonucleosides (Reichard, 1988). While these enzymes are called salvage enzymes the role they play may be intimately involved in maintaining a balance of deoxyribonucleotides in concert with 5' nucleotidases, phosphorylases and deaminases. Mammalian cells contain two sets of deoxyribonucleoside kinases; one in the cytosol and the other in the mitochondria. A deoxycytidine kinase phosphorylates primarily deoxycytidine but also can act on deoxyadenosine and deoxyguanosine (Sarup and Fridland, 1987). Thymidine kinase (tk) is the best characterized

deoxyribonucleoside kinase; it has complex kinetics, dTTP inhibiting tk while dTMP is stimulatory (Cheng, 1978). This enzyme is also able to use deoxyuridine and bromodeoxyuridine (BrdUrd) as substrates. Three separate monophosphate kinases catalyze in mammalian cells the phosphorylation of both ribo- and deoxyribonucleoside monophosphates for adenine, guanine, and cytosine (Reichard, 1988). A previously mentioned dTMP kinase phosphorylates both dTMP and dUMP (Kielley, 1970). This enzyme is a convergent point for *de novo* and salvage synthesis of thymidylate nucleotides destined for DNA replication.

To complete the substrate cycling requires the 5' nucleotidases to dephosphorylate the deoxynucleotides for ultimate export from the cell. One 5' nucleotidase shows specificity for deoxyribonucleotides, with others catalyzing the dephosphorylation of both ribo- and deoxyribonucleotides. To round out the cycle deaminases and phosphorylases break down the nucleosides further and draw the flux toward catabolism when DNA synthesis ceases. The importance of these enzymes is seen in humans with genetic loss of purine nucleoside phosphorylase or adenosine deaminase leading biochemically to an accumulation of dATP or dGTP and in turn to severe immune deficiency (Iizasa and Carson, 1986).

Although deoxynucleotide pools can be measured, they reflect only a static picture of the dynamics and flux of the metabolism of deoxynucleotides. Work done on the kinetics of isotopically labeled nucleosides equilibrating with various dNTP pools has revealed some interesting and unexpected results. The majority of the dTTP pool is derived from the deamination of dCMP and therefore from CDP via RR (Bianchi and Reichard, 1987). In exponentially growing 3T6 mouse fibroblasts one-quarter of the newly synthesized dCDP is eventually excreted, mostly as deoxyuridine (Nicander and Reichard, 1985). A complete block of DNA synthesis by either aphidicolin or arabinosyl cytosine only slowed down dNTP synthesis and led to deoxynucleosides being excreted into the medium. The flux of deoxyribonucleosides can be seen in the other direction with inhibitors (e.g., hydroxyurea) of ribonucleotide reductase. Deoxynucleotides are transported from the medium into the cells and the turnover of dNTPs decreases. All of these observations support the Reichard model of substrate cycling as a mechanism to fine tune the levels of deoxyribonucleotides intracellularly.

I.3 Ribonucleotide Reductase - The Hub of Deoxyribonucleotide Synthesis

Ribonucleotide reductases (RR) have been characterized from a number of different sources and differ a great deal in structure, cofactor requirements, and substrate utilization (e.g., nucleoside diphosphate vs nucleoside triphosphate). On the other hand, all RRs have a reaction mechanism that involves an unpaired electron or free radical. A single active site can reduce purines and pyrimidines, and substrate specificity is modulated by allosteric effectors in most RRs studied. RRs can be categorized into four groups to date: (1) binuclear iron center enzymes, (2) adenosylcobalamin enzymes, (3) binuclear Mn center enzymes, and (4) anaerobic RR from E. coli. The group 1 enzymes will be discussed at some length, so I will give a cursory discussion of the other three types at this point:

Adenosylcobalamin-Requiring RRs

These are the simplest RRs known and contain only a single polypeptide subunit which can vary in size from 76 kDa to greater than 500 kDa. The prototypical enzyme studied comes from Lactobacillus leichmannii (Eriksson and Sjoberg, 1989) and requires nucleoside triphosphates as substrates (Ludwig and Follman, 1978). The mechanism appears to involve a transient AdoCbl free radical which is similar to the iron center RR enzymes.

Manganese-Requiring RRs

rNTPs versus the rNDPs are the substrates for this enzyme, which is found in certain gram-positive bacteria (Schimpff-Weiland et al., 1981). Since hydroxyurea (HU), a potent inhibitor of the group 1 RRs, also inhibits this group, it is thought that Mn^{2+} serves a similar role as Fe^{3+} in the iron requiring RRs. These RRs are able to reduce both nucleoside di- and triphosphates to the corresponding deoxynucleotides (Willing et al., 1988). The recently isolated manganese requiring enzyme from Brevibacteria ammoniagenes is a trimer of the structure ab_2. The spectroscopic date suggest a binuclear manganese center analogous to the binuclear iron center found in the group 1 ribonucleotide reductases (Stubbe, 1990).

Anaerobic RRs

A possible link in the evolution of life from the "RNA world" to the "DNA world" may be lie with the anaerobic RR. This transition would have occurred under low oxygen conditions and therefore would require an anaerobic RR. Genetic evidence had previously suggested that the normal aerobic RR was not required for growth of E. coli under anaerobic conditions (Barlow, 1988). Extracts from anaerobically grown bacteria contain a reducing activity capable of converting CTP to dCTP and are not inhibited by antibodies raised against the aerobic enzyme (Fontecave et al., 1989). After fractionation, the activity required at least five components, two heat-labile protein fractions and several low molecular weight fractions including S-adenosylmethionine (Eliasson, et. al., 1990). In addition one of the open reading frames in the coliform bacteriophage T4 encodes a protein that has homology with the N-terminal amino acid sequence of the E. coli anaerobic RR.

Iron-Requiring RRs

This group of RRs is by far the most thoroughly studied and characterized of the four groups discovered to date. The prototypical enzyme of this group of RRs is the E. coli reductase. This enzyme contains four polypeptide chains in the holoenzyme -- two identical chains make up the small subunit (R2), while the large subunit (R1) is made up of another two identical protomers. The two homodimers come together to form an active heterotetramer. To date there has been no report in the literature of the actual K_d for the dissociation of the large and small subunits of E. coli RR, although it is known that the two subunits do not copurify on any gel filtration system. Perhaps the best indication of the catalytic competent quaternary structure of the iron-requiring RRs is the analogous bacteriophage T4 RR, which forms a tightly complexed 230-kDa heterotetramer composed of two R2 polypeptides of 43.5 kDa and two R1 polypeptides of 84 kDa (Berglund,1975.)

Reaction Mechanism

A direct replacement of the hydroxyl group by a hydrogen occurs at the 2' position of the ribose. Evidence from Stubbe and Graslund indicate that there is an abstraction of the 3'-hydrogen of the substrate that is mediated by a protein radical. Studies by several Swedish groups have resulted in the best characterized stable protein radical to date. The E. coli RR has the radical formed on tyrosine 122 of the small subunit. A binuclear iron center made up of two nonequivalent irons coupled through a μ oxo bridge is the cofactor located near the single tyrosyl radical (Stubbe, 1989) (Figure I-4). Stopped-flow absorption spectroscopy and freeze-quench EPR spectroscopy

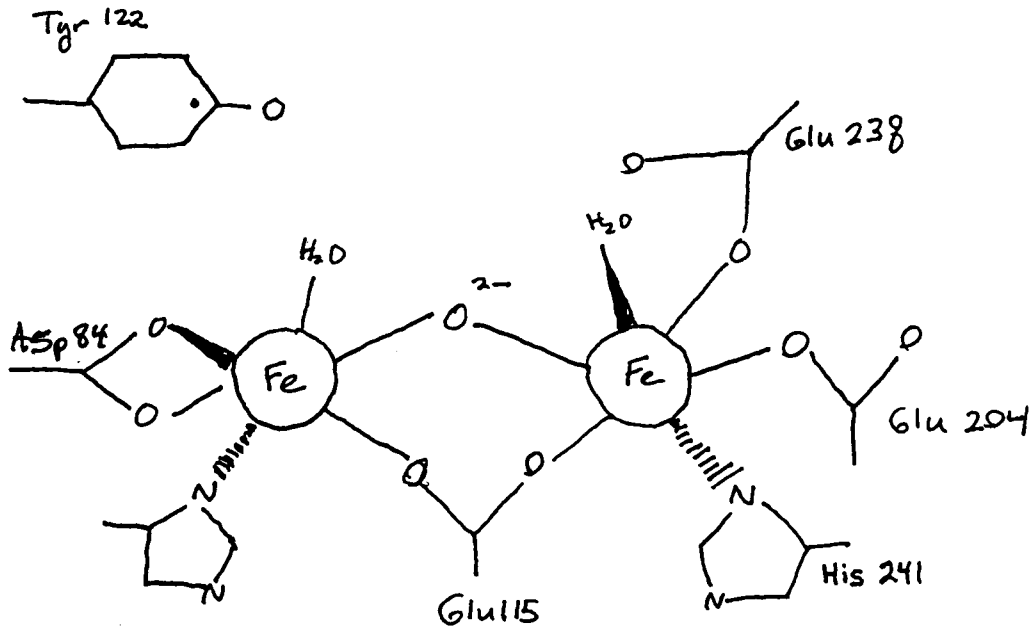


Figure I-4 Iron-center of E. coli ribonucleotide reductase small subunit

has allowed the identification of two novel iron-coupled radical intermediates in the formation of the tyrosyl radical (Bollinger et al., 1991). This mechanistic model is supported by the increased lifetime of the intermediates in a mutant B2 in which the oxidizable tyrosine 122 is changed to a phenylalanine. Recent x-

ray structure data for R2 shows that Y122 is 10 A from the nearest surface, negating the previous assumption that the stable radical was directly responsible for the initial abstracting of a hydrogen from the 3' position of substrate (Nordlund et al., 1990). Stubbe proposes that the reaction proceeds through radical intermediates (Stubbe et al., 1983). A reduction of a radical cation intermediate within the active site by one-electron transfers allows the stereospecific introduction of hydrogen to the 2'-position on the ribose ring (Figure I-5). This entire sequence leaves the R1 subunit with an active site disulfide which needs to be recycled before the next substrate can be acted upon.

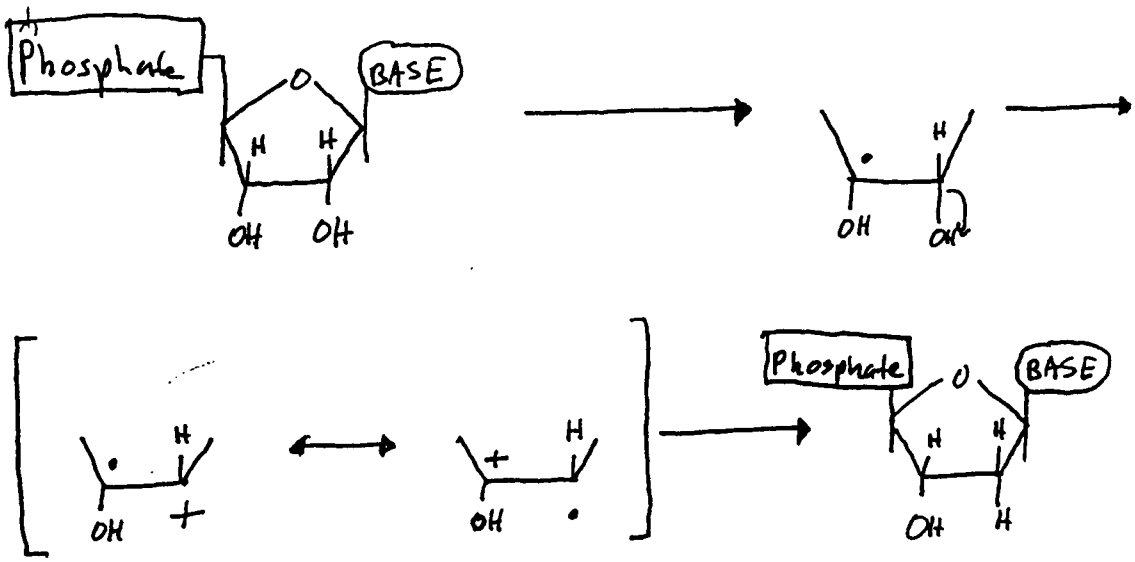


Figure I-5 Reaction mechanism of ribonucleotide reductase

Hydrogen Donor Systems

Dithiols can serve as direct hydrogen donors for purified RR, but there are two known potential hydrogen donor systems; thioredoxin and glutaredoxin, and possible a third in vivo (Holmgren, 1989). While NADPH is the ultimate

hydrogen donor several proteins may participate as hydrogen carriers (Eriksson and Sjoberg, 1989 Figure I-6).

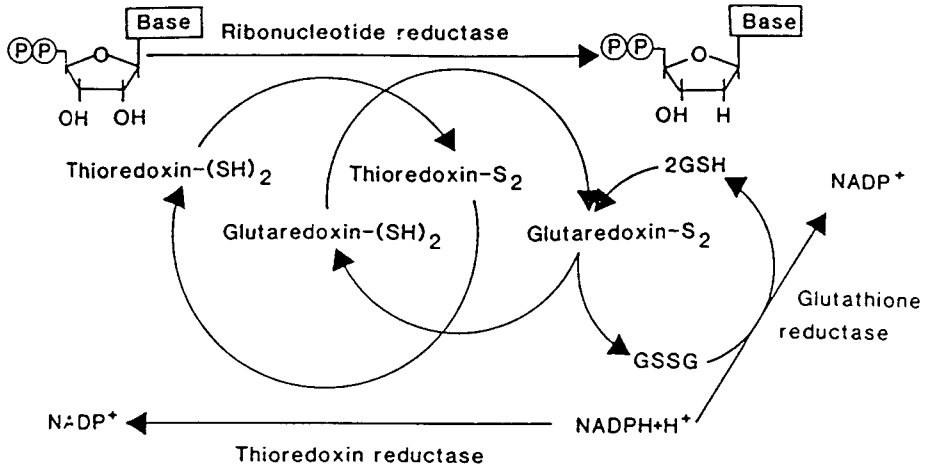


Figure I-6 Hydrogen donor systems

Thioredoxin in *E. coli* is thought to be the primary electron donor, while T4 encodes for a thioredoxin which shares some structural simularities with *E. coli* thioredoxin and is thought to act on the RR from T4 bacteriophage (Holmgren et al., 1986). The primary sequences for T4 and *E. coli* thioredoxin are not homologous, but the tertiary structures are quite similar (Holmgren, 1985). Ironically the T4 thioredoxin and *E. coli* glutaredoxin are homologous proteins. Mutants in *E. coli* lacking thioredoxin (Mark, et al., 1976) were viable and pointed toward the potential use of glutaredoxin as the hydrogen donor for RR. A double mutant for both glutaredoxin and thioredoxin requiring cysteine for growth on minimal media (Russel, et al., 1988) implies the existence on a third hydrogen donor for deoxyribonucleotide synthesis. Vaccinia virus, which encodes the two subunits of RR, also has a gene which has greater than 40% amino acid identity with other known mammalian glutaredoxins. A highly

conserved redox-active site consisting of an amino acid motif of C P F (or Y) C is also found in the vaccinia sequence as well as (Johnson et al., 1991). It remains to be seen if the primary function of this protein is the hydrogen donor for RR or another implicated function of glutaredoxin and thioredoxin. In contrast, a cellular thioredoxin is thought to be the principal hydrogen donor for the herpes simplex virus type 1-encoded RR (Darling, 1988).

I.3.7 Allosteric Regulation

The *E. coli* RR is finely regulated by feedback from dNTPs to determine the substrate specificity as well total activity. The complex regulation of RR is apparently dependent upon the existence of two different allosteric sites (Thelander and Reichard, 1979). The activity site binds ATP or dATP and regulates the overall activity of the enzyme. A separate specificity site binds deoxyribonucleoside triphosphates and ATP and determines substrate specificity. The substrate specificity can be viewed as a cascade to decrease the flow of the overabundant dNTP and increase the production of the underrepresented dNTPs before major perturbations occur. Table I-2 shows the various stimulations and inhibitions of the effector dNTPs on RR. The role of each of these sites in the *E. coli* aerobic RR was originally proposed by Brown and Reichard in 1969 (Brown and Reichard, 1969a). They found that the large subunit of RR contained all the effector sites with two of each class of site found on the dimer R1 subunit. A series of competition experiments between nucleotides showed that dTTP, dGTP, dATP, and ATP could all bind at high affinity sites (h-sites), while only dATP and ATP bound to the low affinity sites (l-sites). There also appeared to be interactions between the two classes of sites as seen by differences in the binding of dATP at the l-site depending upon the nucleotide bound at the h-site. In addition, the sites within a class also seem to interact by showing a decreased binding of the second site nucleotide. Sucrose gradient sedimentation experiments demonstrated that the quaternary structure is dependent upon the effectors present (Brown and Reichard, 1969b).

The prototypic mammalian RR demonstrates similarities to the *E. coli* RR (Engstrom et al., 1979). In solution the R1 behaves as a monomer when no nucleotides are added (Thelander et al., 1980). With dTTP the R1 sediments as a dimer, while the addition of dATP induces the formation of a tetramer. A mixture of dimers and tetramers results when ATP is present. Equilibrium

Table I-2 Regulation of the activities of *E. coli* ribonucleotide reductase. (derived from Mathews and van Holde, 1990)

Nucleotides Bound at			
Activity site	Specificity site	Increase reduction of	Decrease reduction of
ATP	ATP or dATP	CDP, UDP	
ATP	dTTP	GDP	CDP, UDP
ATP	dGTP	ADP	*
dATP	any effector		all four substrates

*Pyrimidine nucleotide reduction is inhibited by dGTP binding to the specificity site of the mammalian enzyme.

dialysis indicated the presence of two classes of effector binding sites, one specific for ATP and dATP, while the second binds dTTP and dGTP in addition to ATP and dATP. Scatchard plot analysis indicated that there is one of each class of binding site per dimer of R1. As a whole the data on the regulation of enzymatic activity of type 1 RR suggests the existence of several conformational states to balance the supply of dNTPs to the replication fork. A composite cartoon of the features of the prototypical iron-containing RR is found in Figure I-7.

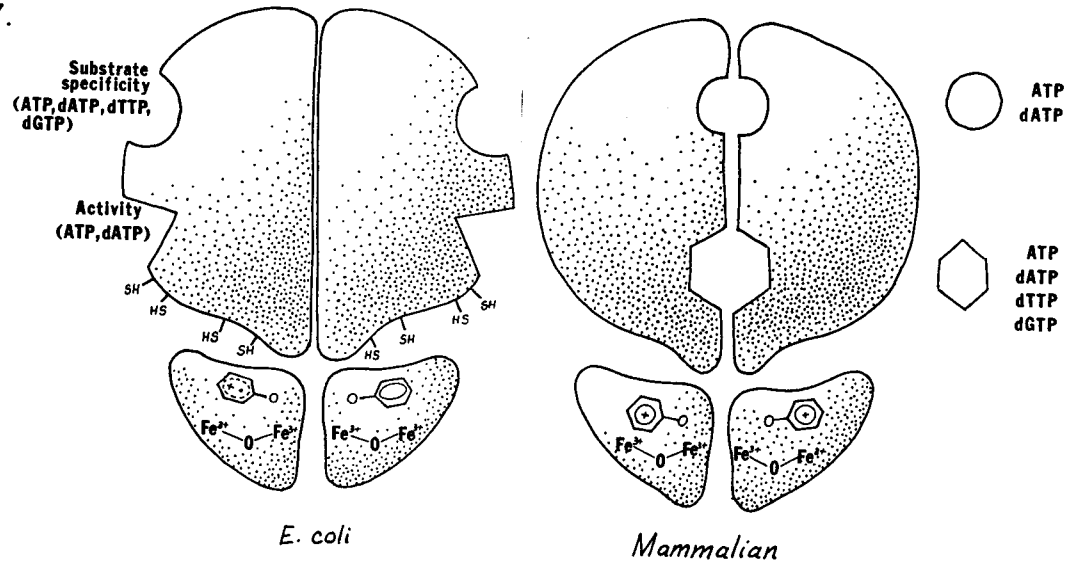


Figure I-7 Schematic representation of important features of RR

I.4 Protein Machines

The main focus of my efforts has been toward probing for the evidence of interactions of RR with other proteins. One perplexing inconsistency of RRs studied in vitro is the seemingly insufficient turnover number for the enzyme to provide enough deoxynucleotides to supply replication forks in vivo. It becomes obvious that some piece of the machine is missing for the efficient coordination of precursor biosynthesis and DNA replication. While it has been the role of biochemists to fractionate a cell extract until only a single activity is present, the conditions under which the homogeneous activity is measured are a far cry from the in vivo conditions. One line of evidence suggests that conditions in a living cell are not like an aqueous bag of proteins, but rather more like a liquid crystal in which there is very little free water to solvate molecules. Diffusion rates of proteins have been calculated to be three orders of magnitude slower than in dilute aqueous conditions (Goodsell, 1991; Brass et al., 1986). In such a scenerio high protein concentrations and little free water can drive interactions of specific proteins with weak association in dilute solutions. Protein crystals, which normally contain around 40% water (Matthews, 1968), can mimic the in vivo situation more closely than the dilute protein solutions usually studied. The protein molecules within crystals are loosely packed, making few contacts with each other, while the space between them is about half water of hydration and half bulk water in its physical and chemical properties. It seems reasonable that since protein crystals can form with as little as 10% protein by weight that water within a cell is present in two phases. This has been verified by two different experimental systems using different methods (Fulton, 1982). In most cases the equilibrium properties of proteins in crystals and in solutions are the same while the kinetic properties of proteins in crystals are altered. Perhap the most accurate description of the in vivo milieu would be as a gel-like substance where the diffusion of metabolites and macromolecules is impeded by other macromolecules and structured water.

Evidence for Interactions of 'Soluble' Enzymes

Experiments of in situ centrifugation using Euglena cells (Kempner and Miller, 1968) and Neurospora (Zalokar, 1960) demonstrated that virtually no macromolecules are in the cytosol layer. These experiments indicate that

proteins in vivo may be all complexed to higher levels of organization. When standard cell fractionation procedures are used to isolate the "soluble phase" many enzymes are found uncomplexed. The phase reference technique allowed investigators to follow intracellular diffusion. By injecting gelatin into a cell as a reference the motion of solutes can then be followed from the intracellular aqueous phase. The results of these experiments showed that the relative amount of total cellular protein diffusing freely was small compared to the total (Fulton, 1982). Other similar experiments using fluorescently labeled proteins to follow the diffusion process by the "fluorescence recovery after photobleaching" (FRAP) technique showed that the diffusion coefficient was independent of the molecular mass of the protein used. In contrast, a series of fluorescently labeled dextrans of different molecular weights showed the expected dependence of the diffusion coefficient on molecular size (Luby-Phelps, 1985). This evidence, as well as modified electron microscopic techniques which show a cytoplasmic mesh (Gershon, et al., 1985), leads to the conclusion that there exist transient interactions between "soluble" proteins and structural elements.

As Wilson points out, the rate of movement and distribution of a protein will depend directly on the strength of its interactions with both formed organelles and more poorly defined structures, such as the microtrabecular lattice (Wilson, 1988). The structural element can be considered as a potential allosteric effector. Binding to the structural effector may influence function of the bound protein, analogous to a classical allosteric effector altering the kinetic properties of an enzyme.

Do Enzymes Need to be so Big for Catalysis?

Paul Srere addresses this question in a TIBS article and concludes that the size of an enzyme may be related to having sufficient surface area to interact with specific binding sites for their localization and integration into metabolic complexes. Some chiral catalysts can match the activity and specificity of enzymes for some reactions. This seems to indicate that it is not necassary to have such a large molecule for efficient catalysis. On the other hand, proteins from a wide variety of living cells show small differences in molecular weight distribution; polypeptides between 30 kDa and 50 kDa constitute 50% of the total proteins (Srere, 1984). Srere goes on to argue that

the surface to volume ratio determines the feasible limits of the proteins and potential interactions. With proteins smaller than 10 kDa the surface area would be limited to one weak interaction, while proteins larger than 50 kDa would have to have much larger mass increments to marginally increase the surface area and hence the number of interaction sites.

Evidence in favor of this hypothesis comes from the experimental results obtained by McConkey using labeled proteins from both human and hamster cells (McConkey, 1982). High-resolution two-dimensional polyacrylamide gel electrophoresis showed that at least half of 370 denatured polypeptides from these two cells are indistinguishable in terms of isoelectric points and molecular weights. Comparing E. coli and HeLa cell proteins showed that 98% of these two sets of proteins are distinguishable and that the overlap of the hamster and human proteins is not just due a multiplicity of proteins. McConkey goes on to propose that evolution was more conservative than expected due to the complexities of intracellular organization and the macromolecular interactions in which many polypeptides are involved in. It is pointed out that the empirical fact that a particular quaternary structure is in the "soluble" fraction may be in large part due to the "cataclysmic violence" of even the most gentle homogenization procedure. The term "quinary structure" is used to include molecular complexes that are transient in vivo.

Complexes of Sequential Metabolic Enzymes

Paul Srere reviewed the concept of complexes of metabolic enzymes in a 1987 review article (Srere, 1987). He points out that within some metabolic pathways there are stable multienzyme complexes and multifunctional enzymes that catalyze sequential reactions. Recent evidence indicates that there are specific interactions among many "soluble" sequential enzymes of metabolic pathways. Table I-3 from Srere's review lists the metabolic pathways in which there is evidence for complexes. A schematic representation of intermediary metabolism presented by Alberts et. al. (1983) represents each intermediate as a black dot and each enzyme as a line (figure I-8). An analysis of the number of dots (table I-4) with only one or two lines connected to it indicates that 80% of the metabolic intermediates have just one use in the cell.

Table I-3 Metabolons (from Srere, 1987)

Metabolic pathway	Evidence*
DNA biosynthesis	A, B, C, E, F
RNA biosynthesis	A, B, C, E, F
Deoxyribonucleotide biosynthesis	A, B, D, E, F, H
Protein biosynthesis	A, B, C, D, F
Glycogen biosynthesis	B, E
Purine biosynthesis	A, E
Pyrimidine biosynthesis	A, B, D, E, F
Amino acid metabolism	A, B, D, H
Lipid biosynthesis	B, C, F, H
Steroid biosynthesis	A, C, E
Glycolysis	A, B, C, D, E, I
Tricarboxylic acid cycle	B, C, D, G
Fatty acid oxidation	A, B, C, D
Electron transport	C, I
Antibiotic biosynthesis	A, E
Urea cycle	B, D
Cyclic AMP degradation	A, D, E

* A = channeling; B = specific protein-protein interactions; C = specific protein-membrane inteactions; D = kinetic effects; E = isolation of complexes or multifunctional proteins; F = genetic evidence; G = model systems; H = existence of multifunctional or multienzymic proteins; I = physical chemical evidence.

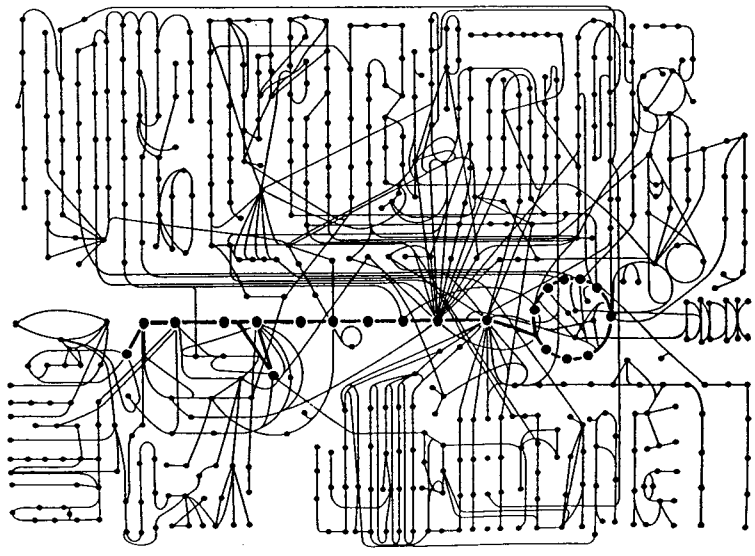


Figure 1-8 Schematic representation of metabolic reactions

Table 1-4 Analysis of metabolic intermediate uses in cell (Srere, 1987)

lines (enzymes)	dots (intermediates)
1 or 2	410
3	71
4	20
5	11
6 or more	8

While all of these systems add to the evidence of weak yet significant interactions, I will focus only on the model T4 bacteriophage system and evidence for eucaryotic DNA polymerases being associated with the nuclear matrix for the background of this thesis.

Bacteriophage T4 DNA Replication

The T4 genome consists of 166 kilobases of double-stranded DNA and codes for over 200 gene products, one half of which are involved in deoxyribonucleotide biosynthesis, DNA replication and the regulation of gene expression during infection. Seconds after infecting the host E. coli immediate early genes are expressed, and a delayed early class of genes are transcribed within two to three minutes. The enzymes and protein factors involved in deoxynucleotide biosynthesis and DNA replication are included in these early genes. By five minutes post-infection T4 DNA replication begins as well as late gene transcription. By 25-30 minutes post-infection approximately 200 mature virus particles are made per infected cell.

In order to meet the high rate of DNA synthesis in phage-infected cells, T4 induces most of the deoxynucleotide biosynthetic enzymes needed to supply dNTPs to the replication fork the phage DNA synthesis (Mathews and Allen, 1983). Even though DNA replication in vivo proceeds at a rate of 850 nucleotides per second per chain at 37° C, the affinity of the DNA polymerase for dNTPs is relatively low. By forming a complex of enzymes to provide a local high concentration of dNTPs at the replication fork T4 effectively couples deoxynucleotide biosynthesis to DNA replication (figure 9).

In addition, the proposed complex can also balance the synthesis of the four dNTPs to reflect the relatively AT-rich T4 genome. While this model predicts that there are two distinct pools of dNTPs, the ability to directly measure the pool at the replication fork is beyond the current technology. Genetic and indirect evidence indicates that there are indeed two pools of dNTPs in the T4 bacteriophage-infected E. coli (Ji and Mathews, 1991). Kinetic coupling of enzymatic activity could be demonstrated in vitro and phage with mutations in T4 ribonucleotide reductase disrupted a rapidly sedimenting "dNTP synthetase complex" (Moen and Mathews, 1988). In addition, recent experiments have shown multiple interactions of immobilized dCMP hydroxymethlase with other dNTP biosynthetic enzymes (Wheeler et al., 1992).

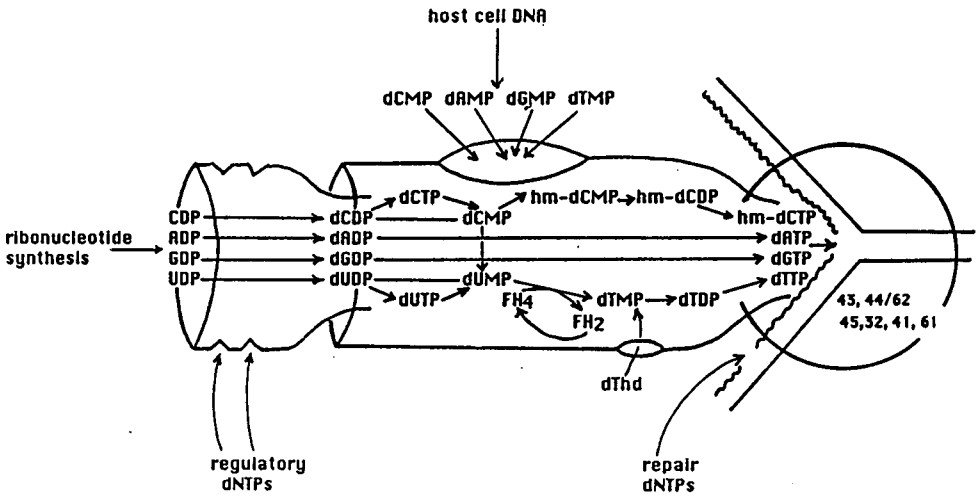


Figure 1-9 Speculative view of the T4 dNTP-synthesizing multienzyme complex (Mathews, 1993)

An interaction between thymidylate synthase and dCMP hydroxymethylase has been detected with the use of anti-idiotypic antibodies and shown to be specific by competition with unlabeled thymidylate synthetase and dCMP hydroxymethylase (Young and Mathews,1992).

Alberts has analyzed the multienzyme complex that is involved in DNA synthesis of the T4 bacteriophage. Seven different purified proteins from individual genes can be reconstituted to synthesize new DNA from a DNA template. The DNA polymerase is thought to participate in multiple discrete types of DNA polymerase complexes. Other polypeptides involved include a helix-destabilizing protein, three polymerase accessory proteins, DNA helicase,

and two proteins for primer synthesis. As such, the DNA-synthesizing multiprotein complex probably does not exist in solution, but is assembled on the DNA template. The DNA can be viewed as serving as a nucleation site for the assembly of the complex as well as a template.

Physical interactions have been detected by using an affinity chromatography technique with immobilized T4 helix-destabilizing protein (gp32). A T4-infected cell extract passed over a gp32 column binds 10 different T4-induced proteins (Formosa et al., 1983). This technique has been extended to affinity purify the T4 DNA polymerase with a gp32 column. The role of gp32 in replication appears to multifaceted and in itself serve perhaps as an assembly point for replication complexes, as indicated by genetic evidence (Mosig et al., 1979) where second site suppressors for gene 32 were characterized, as well as physical and biochemical data.

The DNA chain elongation system of bacteriophage T4 includes T4 DNA polymerase, the genes 44/62 and 45 accessory proteins, and gene 32 single-stranded DNA-binding protein, which are all required for synthesis of the leading and lagging strands (Richardson, et al., 1990). Aside from the tight complex of 44/62, the physical associations among the polymerase, the accessory proteins, and 32 protein are weak. The T4 polymerase accessory proteins are thought to function as a sliding clamp that secures the polymerase to the primer-template. Even the association of the holoenzyme with a primer-template is short-lived (10s at 22°C) when buffer and salt concentration at chosen to mimic *in vivo* conditions (Jarvis et al., 1991). While the physical interactions of the proteins are too weak to allow isolation of the multienzyme complexes, the interaction of the T4 proteins can be readily demonstrated by the effect of one protein, or a set of proteins, on the reaction catalyzed by another (for examples see Richardson et. al. 1990).

A cross-linking technique using a primer with a thymidine analog attached to a photoactivatable aryl azide was utilized to determine the stepwise assembly of the T4 DNA polymerase and its accessory proteins on the primer-template (Capson, et al., 1991). The results of these experiments indicate that there is initial binding of the three accessory proteins and ATP to a gp32 protein-covered primer-template, followed by ATP hydrolysis, binding of polymerase, and movement of the accessory proteins to yield a complex capable of processive DNA synthesis. In addition to increasing the polymerase processivity, the accessory proteins stimulate duplex unwinding by the T4 gene

41 helicase and are involved in the transcription of the late T4 genes (Herendeen, et al. 1989). Although beyond the scope of this thesis, the T4 replication complex without the polymerase provides an example of a replication complex acting as a mobile enhancer and may involve further transient interactions between protein complexes.

Are DNA Polymerases Attached to a Structural Element?

The most widely held model of DNA replication says that soluble polymerases bind to the "origin" and then track along the template as they synthesize DNA (Alberts, 1990). Recent evidence with eucaryotic polymerases suggests that active polymerases are bound to a skeleton in the eukaryotic nucleus, with replication occurring as DNA is reeled through a fixed complex (Jackson, 1990). As Cook (1991) points out, if polymerases run "down the template track", the expected sites of replication would be scattered diffusely throughout the nuclei and would represent the template concentration. In contrast, when rat fibroblasts are pulse-labeled with bromodeoxyuridine during S phase and sites of incorporation are then visualized with fluorescently labeled antibodies against the nucleotide analog, discrete foci can be seen scattered throughout the nuclei (Nakamura et al., 1986, Nakayasu and Berezney, 1989). Another series of experiments, which is subject to numerous artifacts due to the nonphysiologically high salt concentrations, showed that nascent DNA was tightly associated with the nuclear "matrix" (Berezney and Coffey, 1975). By encapsulating HeLa cells in agarose microbeads before lysing membranes in a "physiological" buffer (Jackson and Cook, 1985; Jackson et al. 1988) the resulting encapsulated nuclei can be treated with an endonuclease and the bulk of the chromatin removed by electrophoresis. Essentially all the DNA polymerizing activity is recovered in the residual clumps of chromatin which is attached to a skeleton of the nucleus. A soluble polymerase is also found but its activity does not change during the cell cycle (Jackson and Cook, 1986)

If such complexes are indicative of the in vivo situation, how do in vitro replication systems work? The answer is very poorly; systems are efficient when crude and become inefficient when highly purified. The SV40 DNA in vitro system seems to belie this point at first glance (Wobbe et al. 1987; Ishimi et al., 1988; Tsurimoto et al., 1990) in that there are no skeletal elements. Cook

comes to the conclusion that the T antigen which is used at very high concentrations in these replication systems is partially or completely immobilized to form an artificial skeleton *in vitro.*

I.5 The System Used for Probing Interactions Involving Ribonucleotide Reductase--Vaccinia Virus

From the previous section it can be concluded that just because a protein can be isolated from a soluble extract and fractionated to the point of apparent homogeneity does not guarantee that the protein functions in the same manner as it would *in vivo.* While T4 bacteriophage has provided the prototype for exploring such interactions of deoxyribonucleotide and DNA synthesis, an analogous system in the eucaryotic world would be desirable. While eucaryotic cells have a certain attractiveness for such studies and have indeed been pursued with varying results by others (for a review see Mathews, 1992), the complexity and genetics of such systems make their manipulation difficult and very time consuming. On the other hand most eucaryotic DNA viruses replicate and utilize the host nucleus for varying degrees of their DNA synthesis process. Poxviruses differ in that they replicate in the cytoplasm rather than in the nucleus. Vaccinia (VV) is the prototypical member of the Orthopoxvirus genus of the Poxviridae family. The double-stranded DNA genome of nearly 200 kilobase pairs potentially encodes most or all proteins which are required for replication and transcription in the cytoplasm. The haploid genetics of VV is a simplifying advantage of the viral system, especially now that the entire genome of VV is sequenced (Paoletti, 1991) and foreign DNA can be inserted relatively easily into the genome (Miner and Hruby, 1990). Virus replication and assembly are localized to discrete foci in the cytoplasm of infected cells. The term virosome or virus factory has been used to describe the inclusion bodies seen in electron micrographs of infected cells. As will be demonstrated in this thesis, unambiguous localization of the sites of DNA synthesis can be demonstrated by the use of a thymidine analogue to pulse-label these sites for detections by immunocytochemical means.

Overview of the Molecular Biology of Vaccinia Virus

The vaccinia virus infectious cycle is shown in the cartoon in Figure 1-10. Upon infection a transcription system which is specific for early genes is activated in a complex core structure. All the proteins necessary for early gene transcription are carried in the virion: a multisubunit DNA-dependent RNA polymerase, an early transcription factor, capping and methylating enzymes, and a poly (A) polymerase. The early RNAs are capped, methylated, polyadenylylated, and are terminated about 50 nucleotides after the sequence TTTTTNT. To date there has been no reported splicing of vaccinia transcripts. The early RNA is extruded from the viral cores and translated in the cytoplasm of infected cells. The early genes transcribed include the RNA polymerases subunits, enzymes required for DNA replication, and putative factors for transcription of intermediate genes.

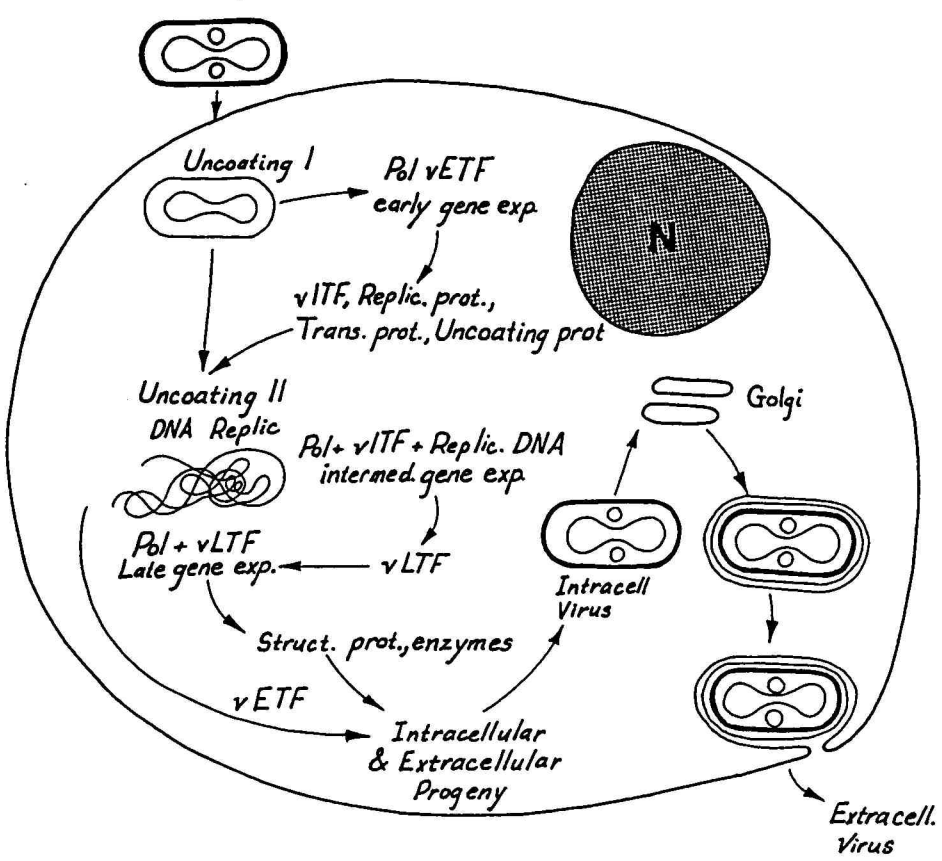


Figure I-10 Overview of vaccinia virus lifecycle

The viral cores are uncoated and dump the viral DNA into the discrete locations as seen in electron micrographs. The virion DNA must become accessible to the newly synthesized vaccinia DNA polymerase. The replicated DNA is accessible for the transcription of intermediate genes, which include three open reading frames (ORFs) identified as trans-activators of late gene expression. The last phase of gene expression includes the expression of late genes encoding structural proteins, virion enzymes, and early transcription factors. VV late RNAs have no well defined 3'-ends and also contain a 5' poly(A) leader (Weir and Moss, 1984; Schwer et al. 1987) Viral structural morphogenenesis occurs at this stage and is thought to have some role in the inhibition of the transcription of early genes again.

While regulation of vaccinia genes has been at the forefront of recent explorations of the infectious-cycle of poxviruses, there are indications that regulation occurs at the level of translation, RNA turnover, feedback inhibition, and post-translational modification (VanSlyck and Hruby, 1990).

Vaccinia DNA Replication

Even though other phases of the molecular biology and biology of vaccinia are integral aspects of virus replication, synthesis of the precursors for DNA replication and the potential interactions of the proteins involved are the focus of this thesis. Due to the inhibition of host DNA synthesis, transcription, and translation, the proteins involved and the synthesis of viral DNA can be followed readily. The onset of DNA replication occurs before 2 hours post infection at multiplicities of infection of 15 plaque forming units (pfu's) per cell. In earlier studies DNA synthesis was measured by the incorporation of exogenously added [^{3}H]-thymidine into TCA-precipitable counts. The peak thymidine incorporation was observed between two and four hours post-infection, with little detectable synthesis after 5 hours. However, measuring DNA synthesis by quantitative filter hybridizations showed that DNA accumulates at a constant rate until 10-12 hours post-infection (Ensinger, 1987; Hooda-Dhingra et al. 1989, Rempel et al 1990). This discrepancy is probably due to the complex regulation of thymidine kinase and intracellular nucleotide pools. Although vaccinia DNA can accumulate to half the nuclear DNA content,

only about 30% of these sequences are encapsidated (Joklik and Becker, 1964).

Replication Proteins Identified

Temperature-sensitive mutants have allowed for the identification of several replication proteins. Three complementation groups which can express early genes but not synthesize viral DNA at the non-permissive temperature have been found: DNA polymerase; D5, a 90-kDa protein of unknown function (Evans and Traktman 1987, Roseman and Hruby, 1987); and B1, a 34-kDa protein kinase. Very little is known about the mechanisms of vaccinia DNA replication, such as the site of initiation or whether synthesis proceeds by only leading strand synthesis. It does seem that more of the proteins involved need to be identified to fill in the picture. At the very least an endonuclease, elongation accessory proteins for the DNA polymerase, and a helicase in addition to the DNA binding protein described in this thesis will be neccessary to complete the replication system.

Vaccinia Virus Deoxynucleotide Metabolizing Enzymes

At the time this thesis was started only thymidine kinase was definitively mapped on the VV genome (Hruby and Ball, 1983). In the intervening period a dUTPase (Roseman, unpublished results), which was originally thought to be a pseudoprotease (Slabaugh and Roseman, 1989), and a thymidylate kinase (Hughes et al., 1991) have been demonstrated to be functional genes. Both subunits of ribonucleotide reductase have been mapped, sequenced (Slabaugh et al., 1988; Tengelsen et al., 1988) and the cloned recombinant proteins shown to be enzymatically active (Howell et al., 1992). Proteins with significant homologies for thymidylate synthase, dihydrofolate reductase or nucleoside diphosphokinase have not been found in the genome of the Copenhagen strain of VV, which been entirely sequenced.

I.6 Overview of the Questions Asked

The work in this thesis centers on vaccinia ribonucleotide reductase and potential interactions in infected cells. While the work is not exhaustive of the potential techniques to explore the question of higher levels of organization there were several different approaches taken. The first section involved initially establishing that VV does indeed code for RR and determining the primary sequence of the small (R2) subunit polypeptide The second section utilizes techniques which do not always yield clear cut results, but sometimes suggest a higher level of *in vivo* organization. Finally the third section defines a specific interaction by anti-idiotypic antibodies.

Chapter II
Mapping and Sequencing the Vaccinia Ribonucleotide Reductase Small Subunit Gene

II.1 Background

Dr. Mary Slabaugh in this laboratory had previously found that vaccinia-infected BSC_{40} cells showed an increased ribonucleotide reductase activity (Slabaugh et al. 1984). The activity was studied in cell-free extracts from mock-infected and infected monkey kidney cells. There was a 20-30 fold increase in activity at high multiplicities of infection (m.o.i = 50 pfu/cell) and the magnitude of the increase was dependent on the multiplicity of infection. A time course experiment demonstrated that the activity increase was transient. The activity peak was at 4 to 5 hours post-infection, corresponding to the time of maximal viral DNA synthesis as determined by pulse-labeling DNA with [^{3}H]-thymidine. Inhibition of DNA synthesis increased and prolonged the induced activity, a result consistent with the idea that the RR genes would belong to the early class of vaccinia genes. The induced RR activity is distinct from that induced by another DNA eucaryotic virus, herpesvirus, as shown by sensitivity to the allosteric regulators dATP and dTTP. Both regulators inhibit CDP reduction by the host reductase and vaccinia reductases while herpesvirus is relatively insensitive to these metabolites. Differences between the induced vaccinia activity and host activity of RR were observed for adenylylimido-diphosphate (AMP-PNP) stimulation, Mg^{2+} optimum, and K_m (CDP) strongly suggesting that the induced activity was a different enzyme from that of the host (Slabaugh and Mathews, 1984).

To obtain more evidence for a viral RR, Dr. Slabaugh isolated strains of VV which were resistant to hydroxyurea (HU) (Slabaugh and Mathews, 1986). This drug was chosen because it inactivates the free radical which is required for enzyme activity. A rapid depletion of deoxynucleotides will subsequently halt DNA synthesis, while showing little effect on RNA and protein synthesis. Repeated passages of VV in hydroxyurea yielded isolates capable of growing at a hydroxyurea concentration (5 mM) which inhibited wild-type VV 1,000-fold. These mutants showed an overproduction of a 34-kDa viral protein starting early in infection. Assays of RR activity showed a 4- to 10-fold increase over wild type infections. The mutants incorporated [^{3}H]-thymidine into DNA earlier

than wild type if grown in the presence of HU. Given the fact that the target of HU is the small subunit of RR and the size of the overproduced 34 kDa protein was in the size range of known polypeptides for R2, Dr. Slabaugh's results suggested that the vaccinia virus encodes a gene for R2.

At this point in the project, I joined the laboratory and contributed to the mapping and sequencing of the R2 gene in vaccinia virus. Much of my work was of a joint nature with Dr. Slabaugh's research. My major contributions were: 1) Gross mapping of two of the HU mutants to left end of the *Hin* d III F restriction fragment of the vaccinia genome, and 2) subcloning all of the fragments used for sequencing, and 3) sequencing all of the R2 gene on one strand and approximately 30% of the other.

II.2 Materials and Methods

In general, all the protocols used for molecular biology techniques are found in the latest edition of Molecular Cloning (Sambrook et al., 1989).

Preparation of Viral DNA

BSC_{40} cells in 100-mm plates were infected with wild-type (wt) VV (0.1 PFU per cell) or hydroxyurea-resistant mutant HU1 or HU19 (1 PFU per cell). 5mM hydroxyurea was used when the mutants were grown in the presence of the drug. After 48 hours the viral DNA was purified from the infected cells using a procedure developed by Condit et al. (1983). Digestion of the purified DNA was carried out with 10 units of restriction endonuclease/10 µl purified VV DNA for 2 hours at the manufacturer's (BRL) specified temperature. The digested DNA was run on a 1% agarose/TBE gel system until bromophenol blue marker reached the bottom of the gel and visualized with ethidium bromide staining (5 µg/ml of TBE).

Subcloning of the Left End of VV Hind III F

A clone of the *Hind*III F fragment was originally obtained from B. Moss. A 400 -ml culture of pBR322/*Hind* III F plasmid in JM83 was grown in LB with 85 µg/ml of ampicillin. The culture was treated with 170 µg/ml of chloramphicol at A_{595}= 0.4 and allowed to grow for 16 hours. A RNA-free plasmid preparation was obtained by using a column chromatography protocol developed by Micard et al. (1985). Vectors M13mp18 and mp19, pUC18 and 19 were used as subcloning and sequencing vectors. Restriction sites in *Hind* III F fragments were chosen to utilize the cognate site in the polylinker of the vectors. The total DNA restricted was enough to yield 1 µg of the desired fragment to be subcloned. Both the desired fragment to be subcloned and the vectors were gel isolated in an agarose/TAE electrophoresis system. After staining with ethidium bromide and visualizing with UV light, the appropriate size fragment was excised from the gel and purified using a modification of phenol extractions and subsequent ethanol precipitations. Instead of using low-melting agarose, the agarose slice was mashed and an equal volume of TE equilibrated was added. After a 15 minute incubation at -70° C the slurry was centrifuged at room

temperature for 15 minutes at 15,000 r.p.m. in a microfuge. The remaining steps of the procedure were carried out as described in Maniatis et al. (1982). The fragments were ligated with the vector at an estimated (on a gel) ratio of 3:1 overnight at 15° C and transfected into JM 83 cells for the pUC recombinants or JM 107 for the M13 recombinants. Blue/white screening allowed the initial picking of putative recombinants with mini-preparations of the isolated recombinant DNA confirming that a given colony (or plaque) contained the desired insert piece of DNA (Sambrook et al., 1989).

Template Preparation and DNA Sequencing

M13 phage was isolated from the supernatant of infected JM 107 cultures and purified using standard PEG precipitation procedures (Sambrook et al., 1989). The protein was removed from the DNA by extracting once with equal volume of phenol, once with 1:1 phenol/$CHCl_3$, and once with $CHCl_3$. The DNA was ethanol precipitated and resuspended in TE. 1 µg of single-stranded DNA was annealed with the universal primer (BRL). Either Klenow fragment (New England BioLabs) or Sequenase (USB) was used with the dideoxynucleotide chain termination method of DNA sequencing (Sanger, et al., 1977; Tabor and Richardson, 1987) using [α^{35}S]-dATP (NEN) as the label in the reactions.

Double-stranded DNA sequencing was done on pUC recombinants which had been purified by the Holmes and Quigley (1981) method. 1-2 µg of plasmid DNA was alkali denatured and the universal primers (BRL) were annealed. Sequence from both strands of the pUC recombinant utilizing forward and reverse primers in separate reactions. Initially, reactions were run on a 8% denaturing polyacrylamide gel system using TBE as the buffer with extended runs done on 5% denaturing polyacrylamide gel.

Oligonucleotide Primer

An oligonucleotide RD1(CATAAGTATCGATCAAA) was synthesized by the Central Service Laboratory of the Center for Gene Research and Biotechnology, Oregon State University. The oligonucleotide was gel purified on a 20% denaturing polyacrylamide gel (Ellington, 1987).

II.3 Results

Gross Mapping of HU-resistant Mutants 19-5-3 and 1-5-3.

These were two of the mutants Dr. Slabaugh had chosen for further characterization because of their more stable phenotype. One way to initially analyze mutants is to check for restriction length polymorphisms when the genomic DNA is digested with a battery of restriction endonucleases. Vaccinia virus DNA is easily isolated and extracted away from the viral proteins and can then be digested with different restriction enzyme to yield characteristic band patterns when fractionated by electrophoresis. A number of restriction digest patterns have been published and currently most genes in vaccinia virus are referred to in relation to their location on the *Hin*d III map of VV. The *Hin*d III digestion gives fragments from A (49.5 kb) to P (0.3 kb) with the series proceeding alphabetically from high to low molecular weight. Other restriction enzymes will yield different patterns but with a simular nomenclature.

Initial experiments were done by preparing vaccinia virus DNA from the wild type WR infections. *Hin* d III, *Xho* I, and *Kpn* I were chosen for the initial characterization because there was a well defined map published by Esposito and Knight (1985). After initial confirmation that the strain we were working with showed the published patterns, mutant DNA was prepared from infections with HU-1 and HU-19 in the presence and absence of 5 mM HU. The DNA was digested with *Hin* d III, electrophoresed as before, and the patterns compared to the wild-type run parallel on the same gel (Figure II-1).

The results from these experiments showed clearly a substoichiometric amount of the *Hin* d III F fragment for the mutants grown in the presence of HU without a clear indication of another fragment replacing it. Digests were done with *Eco* R I and no obvious differences were observed at the time. At this time, Dr. Slabaugh had been doing experiments with a degenerate oligonucleotide probe to also locate the gene. A positive signal was seen for both the *Hin*d III D and F fragments of the vaccinia genome (Figure II-2). The data indicated that the R2 gene was probably located in the left end of *Hin* d III F, although *Hin* d III D was not completely ruled out. I shifted my emphasis to subcloning the left ends of *Hin* d III D and F.

Subcloning and Sequencing the R2 gene

The initial sequencing strategy involved generating subclones from the pBR322/*Hin*d III library of vaccinia virus WR strain provided by the Hruby laboratory (OSU, Microbiology). After isolating the pBR322/*Hin*d III VV F plasmid, the DNA was restricted with *Eco* RI and *Hin*d III to generate three cloned fragments from vaccinia genome and the pBR322 vector. During this time Dr. Slabaugh had further localized the part of the gene to which the degenerate oligonucleotide (MS-1) bound, the *Hin*d III/*Eco* R I fragment at the left end of *Hin*d III F. The *Hin*d III/*Eco* R I fragment was gel isolated and subcloned into M13 mp18 and mp19 for sequencing from both the universal primer and the MS-1. My sequencing from the mp19 clone yielded sequence homology with known R2 genes while Dr. Slabaugh concurrently found sequence of R2 from the mp18 clone using oligo MS-1. This information allowed us to orient the gene, to precisely locate the 3' end of the open reading frame and to estimate the location of the 5' end. The general strategy at this point was to sequence from the *Eco* R I site in the opposite direction. Subcloning was completed by utilizing information from the sequence generated and restriction maps from other labs. The middle RI fragment was subcloned into pUC19 and then further subcloned by restriction digestion with *Eco* R I and *Pst* I, or *Pst* I and *Bam*H I. The 300-basepair *Eco*R I/*Pst* I fragment was gel isolated and ligated into pUC19 and M13mp19. Several attempts to obtain the other orientation in M13mp18 failed. The 1.9 kb *Pst* I/*Bam* H I fragment was gel isolated and subcloned into M13mp18 and M13mp19. All sequencing performed with the M13 vectors used the universal primers except for the RD-1 primer which was designed from sequence data obtained. Some of the sequence from the *Eco* R I site to the *Pst* I site was obtained using double stranded sequencing of the pUC19 clones and using forward and reverse universal primers. (Figure III-3)

Approximately 90% of the sequence was acquired off of both strands from this work and sequencing to determine the amplification regions (Slabaugh and Roseman, 1989). The coding portion of the VV R2 gene consists of 957 nucleotides and codes for a protein that is predicted to be Mr 36,943 (Figure II-4). The predicted molecular weight of the R2 polypeptide is compatible with the observed overexpressed polypeptide in the HU resistance

mutants. The ORF is oriented to be transcribed from right to left and is transcribed early in infection as determined by Dr. Nancy Roseman (Slabaugh et al., 1988).

II.4 Discussion

The mutant DNA proved to be an important tool for mapping the gene by restriction digestion. Amplification events are one known mechanism for providing drug resistance. The substochiometric amount of the *Hin*d III F restriction fragment proved to be a heterogenous pool of viral genomes with varying degrees of amplified R2 gene (Slabaugh et al., 1988). Hydroxyurea inhibits ribonucleotide reductase by scavenging the proteinaceous radical on the small subunit of ribonucleotide reductase which ultimately shuts down DNA synthesis. These mutants overcome the block by an increased gene copy number and hence increased R2 protein. The mutants demonstrate near normal amounts of the wild-type *Hin*dIII F fragment when grown without selection.

The vaccinia R2 protein is highly homologous (80%) to the mouse R2 polypeptide, with 258 out of 319 amino acids being identical. Other eucaryotic R2 polypeptides show 71% (clam) and 65% (yeast) homology with the vaccinia R2. A conserved tyrosine at amino acid 108 is probably the location of the tyrosine radical. At the other end of the evolutionary spectrum is T4 RR with a 14% identity. A phylogenetic tree demonstrates the relatedness of R2 polypeptides with three groups being apparent: herpesviruses, eucaryotes, and procaryotes (Figure II-5). The sum of the lengths of the line segments connecting any pair of sequences is proportional to the relatedness based on overall sequence homologies. This indicates that the eucaryotic proteins are more closely related to each other than are the members of the herpesvirus family. The high homology between vaccinia and the host R2 (mouse) suggests possibly a reverse transcription event of the host R2 mRNA led to the incorporation of R2 into the viral genome.

Since the initial sequencing of the small subunit polypeptide of vaccinia ribonucleotide reductase the R1 gene was sequenced and the transcriptional unit determined (Tengelsen, et al., 1988). This conclusively demonstrated that vaccinia virus carried the genes for the holoenzyme. Howell et al. (1992) have shown that the cloned R2 displays the characteristic EPR signal of the tyrosine radical. A mixture of purified cloned R1 and R2 have the ability to reduce nucleoside diphosphates (Slabaugh, personal communication). Even though wild-type vaccinia virus encodes for functional RR, gene inactivation

experiments by Child et al. (1990) and Perkus et al.(1986) have demonstrated that RR is not necessary in tissue culture although these mutants are greatly attenuated *in vivo*.

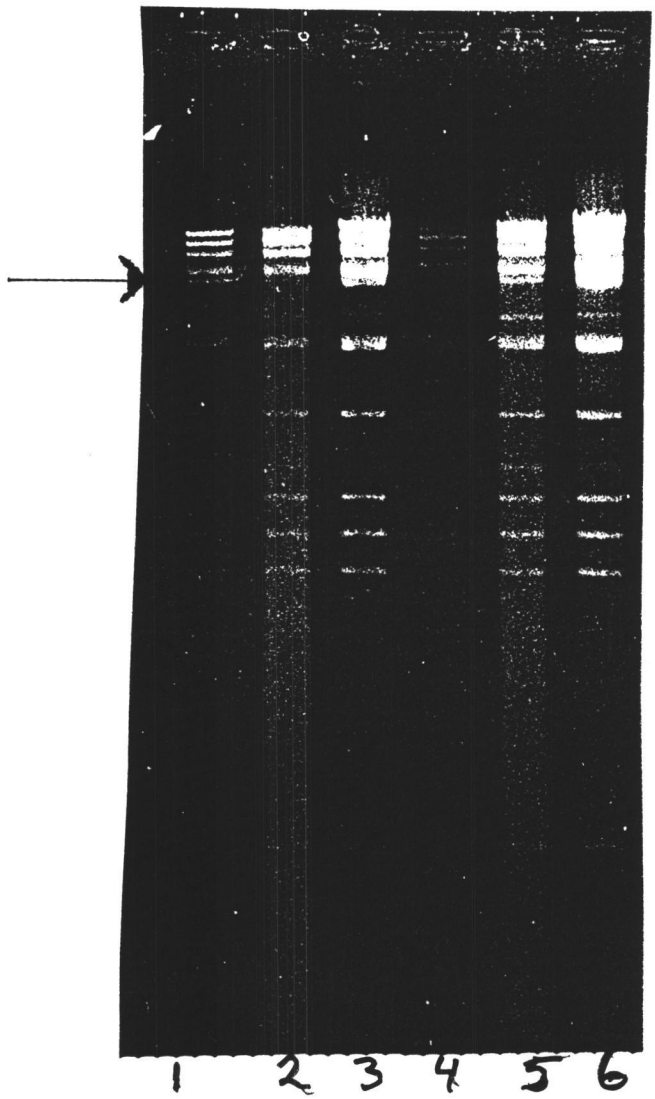


Figure II-1 Restriction analysis of wild type and hydroxyurea-resistant vaccinia virus DNA. All lanes were restricted with *Hin*d III: Lane 1 wild-type DNA, lane 2 mutant 19-5-3 grown in the presence of hydroxyurea, lane 3 mutant 19-5-3 without hydroxyurea, lane 4 mutant 1-5-3 grown in the presence of hydroxyurea, lane 5 mutant 1-5-3 grown in the absence of hydroxyurea. Arrow indicates the *Hin*d III F fragment.

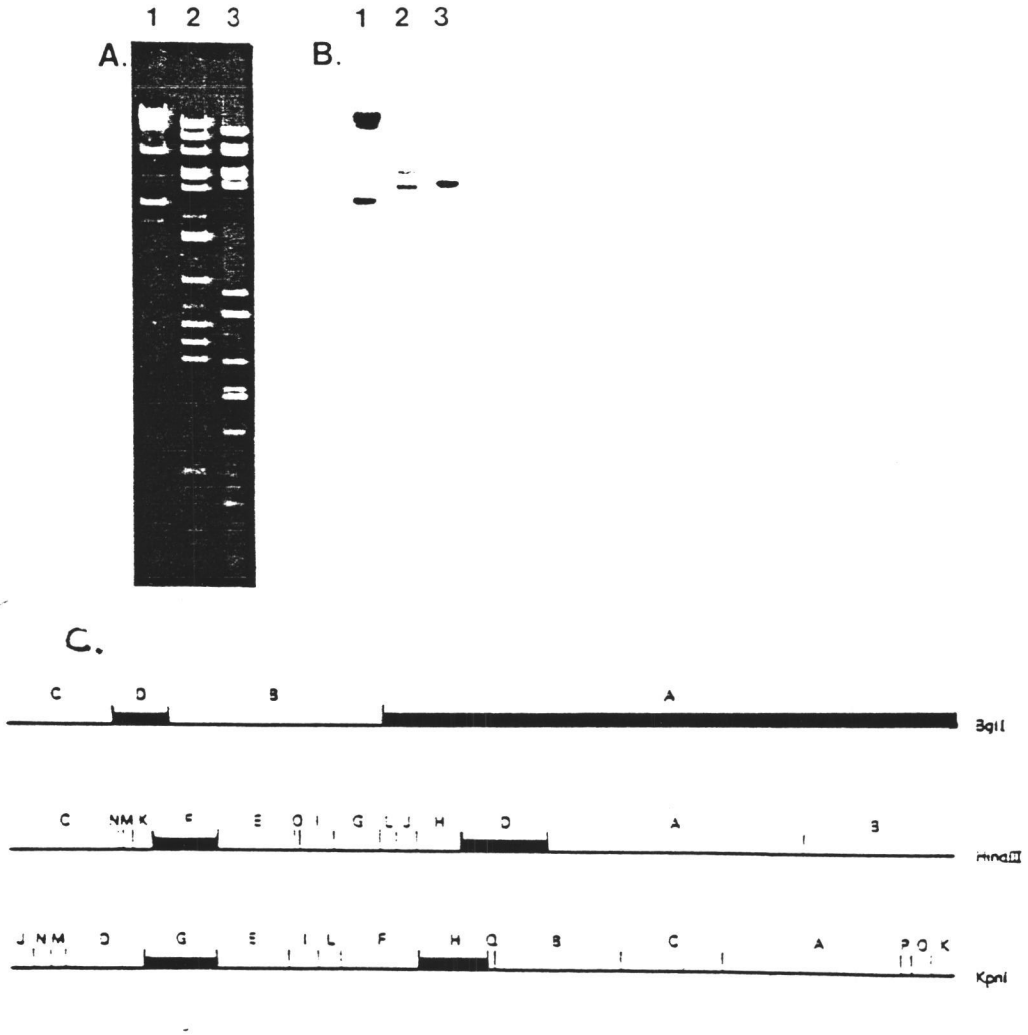


Figure II-2 Mapping of the VV M2 gene using oligonucleotide probes. (A) Stained gel of purified viral DNA digested with *Bgl* I, *Hin* d III, and *Kpn* I (lanes 1 to 3, respectively). (B) Southern blot of the gel shown in panel A probed at 37°C with end-labeled oligonucleotide pool MS1. (C) Diagrammatic representation of hybridization of MS1 to the VV genome. Solid bars indicate hybridizing fragments.

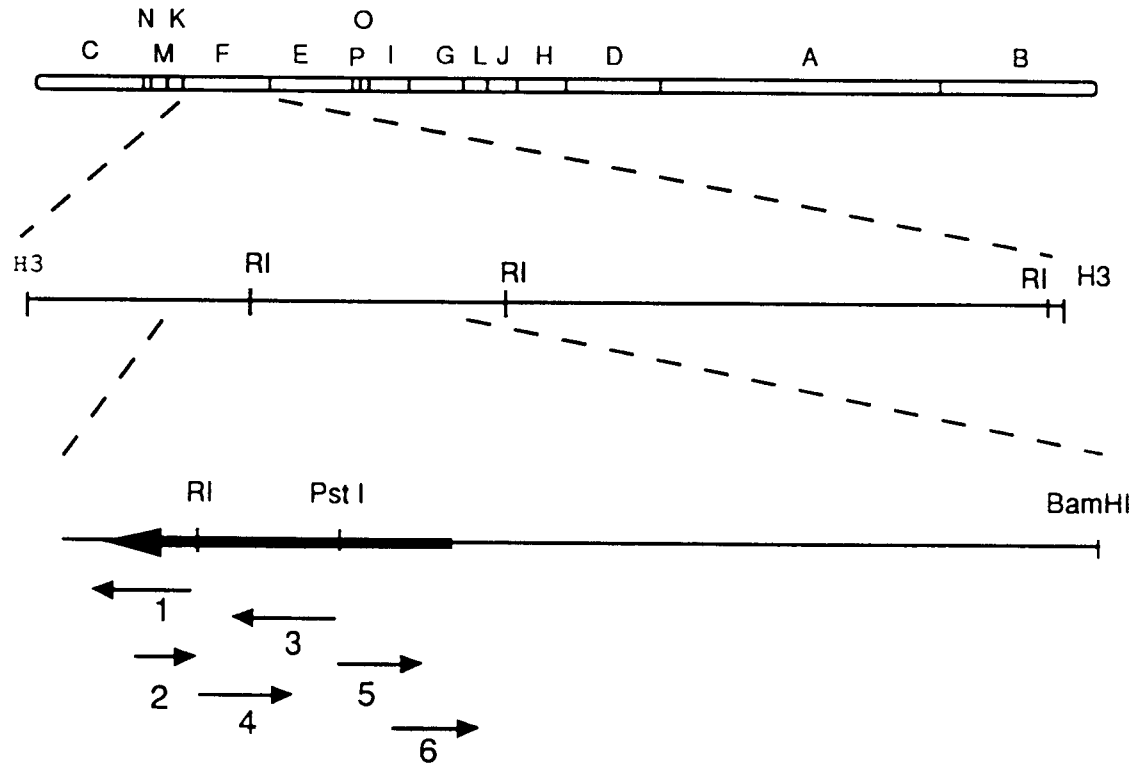


Figure II-3 Sequencing strategy for R2 gene. Diagrammatic representation of subclones for *Hin* d III F fragment of VV genome. RD-1 is the oligonucleotide synthesized to complete the sequencing. The large arrow shows the open reading frame and direction. Small arrows represent sequence derived from a given clone (pUC or M13) and primer.

1. M13mp19 /H3-R1 primer with U primer .
2. M13mp18/H3-R1 with MS-2 primer.
3. pUC19/R1-Pst1 and M13mp19/R1-Pst1 with U primer.
4. pUC 19/R1-Pst1 with reverse U primer.
5. M13mp18/Pst1-BamH1 with U primer.
6. M13mp18/BamH1 with RD-1 primer.

(U =universal)

```
   1 CCA TGA ATG TCG ATA ATT TAA TTA TGA TAG TAC TAA TAA CAA TGC TAT CAA TAA TAC TTG

  61 TAA TTA TTG TAG TGA TTG CGG CGA TAT CTA AGT AGA AAA GAT CCA AGT ACA GGC ATA TAG

                                                    ↓
 121 ATA ACT GAA AAA AAA TTT ATT GTT ATT GTT AAT TTA GTT ATG GAA CCC ATC CTT GCA CCA
                                                    MET Glu Pro Ile Leu Ala Pro

 181 AAT CCA AAT AGA TTT GTT ATT TTC CCA ATC CAA TAT TAT GAC ATC TGG AAC ATG TAT AAA
     Asn Pro Asn Arg Phe Val Ile Phe Pro Ile Gln Tyr Tyr Asp Ile Trp Asn Met Tyr Lys

 241 AAG GCA GAG GCA TCA TTT TGG ACA GTG GAA GAA GTA GAT ATA TCT AAA GAT ATC AAT GAT
     Lys Ala Glu Ala Ser Phe Trp Thr Val Glu Glu Val Asp Ile Ser Lys Asp Ile Asn Asp

 301 TGG AAT AAA CTA ACA CCA GAC GAA AAA TAT TTT ATA AAA CAT GTA TTG GCG TTT TTT GCA
     Trp Asn Lys Leu Thr Pro Asp Glu Lys Tyr Phe Ile Lys His Val Leu Ala Phe Phe Ala

 361 GCC AGT GAC GGA ATA GTG AAT GAA AAT TTG GCG GAA CGA TTT TGT ACA GAA GTA CAG ATT
     Ala Ser Asp Gly Ile Val Asn Glu Asn Leu Ala Glu Arg Phe Cys Thr Glu Val Gln Ile

 421 ACC GAG GCT AGA TGT TTC TAC GGA TTT CAG ATG GCC ATT GAA AAC ATT CAT TCG GAA ATG
     Thr Glu Ala Arg Cys Phe Tyr Gly Phe Gln Met Ala Ile Glu Asn Ile His Ser Glu Met
                 _________RD1_________
 481 TAT AGT CTT TTG ATC GAT ACT TAT GTT AAA GAT AGT AAT GAA AAA AAC TAT CTC TTT AAT
     Tyr Ser Leu Leu Ile Asp Thr Tyr Val Lys Asp Ser Asn Glu Lys Asn Tyr Leu Phe Asn

 541 GCC ATA GAA ACG ATG CCT TGT GTA AAA AAG AAG GCC GAT TGG GCT CAA AAG TGG ATA CAT
     Ala Ile Glu Thr Met Pro Cys Val Lys Lys Lys Ala Asp Trp Ala Gln Lys Trp Ile His

 601 GAC AGC GCC GGT TAT GGA GAG AGA CTT ATT GCC TTT GCT GCA GTA GAA GGA ATC TTC TTT
     Asp Ser Ala Gly Tyr Gly Glu Arg Leu Ile Ala Phe Ala Ala Val Glu Gly Ile Phe Phe

 661 TCT GGA TCA TTC GCT TCC ATA TTT TGG CTT AAA AAG CGT GGC CTA ATG CCC GGA CTC ACG
     Ser Gly Ser Phe Ala Ser Ile Phe Trp Leu Lys Lys Arg Gly Leu Met Pro Gly Leu Thr

 721 TTT TCC AAC GAA CTA ATT AGT AGA GAC GAG GGT CTG CAC TGC GAT TTC GCA TGT TTG ATG
     Phe Ser Asn Glu Leu Ile Ser Arg Asp Glu Gly Leu His Cys Asp Phe Ala Cys Leu Met

 781 TTT AAA CAT TTA TTG CAT CCA CCG AGT GAA GAA ACC GTT AGA TCT ATT ATA ACA GAT GCG
     Phe Lys His Leu Leu His Pro Pro Ser Glu Glu Thr Val Arg Ser Ile Ile Thr Asp Ala

 841 GTA TCC ATT GAA CAA GAA TTT CTT ACT GCG GCT CTT CCA GTT AAA CTT ATA GGA ATG AAT
     Val Ser Ile Glu Gln Glu Phe Leu Thr Ala Ala Leu Pro Val Lys Leu Ile Gly Met Asn

 901 TGT GAA ATG ATG AAA ACA TAT ATA GAA TTC GTC GCG GAT AGA TTG ATT TCT GAA TTG GGA
     Cys Glu Met Met Lys Thr Tyr Ile Glu Phe Val Ala Asp Arg Leu Ile Ser Glu Leu Gly

 961 TTT AAA AAA ATT TAT AAT GTT ACC AAT CCG TTT GAT TTC ATG GAA AAT ATA TCA TTG GAA
     Phe Lys Lys Ile Tyr Asn Val Thr Asn Pro Phe Asp Phe Met Glu Asn Ile Ser Leu Glu
                 _______MS1_______
1021 GGA AAA ACT AAT TTT TTC GAA AAA CGT GTG GGT GAA TAC CAA AAA ATG GGA GTT ATG TCT
     Gly Lys Thr Asn Phe Phe Glu Lys Arg Val Gly Glu Tyr Gln Lys Met Gly Val Met Ser

1081 CAA GAA GAT AAT CAT TTT TCT TTA GAT GTT GAC TTT TAA AGA AAC ATA AAT GCC GAT ATT
     Gln Glu Asp Asn His Phe Ser Leu Asp Val Asp Phe TER

1141 TGT TAA TAC TGT GTA CTG TAA GAA TAT ATT AGC ATT GTC TAT GAC TAA GAA ATT CAA AAC

1201 AAT TAT TGA TGC TAT AGG TGG CAA TAT AAT AGT CAA TTC TAC GAT ATT GAA AAA GTT ATC
```

Figure II-4 Nucleotide sequence of VV DNA encoding the small subunit of ribonucleotide reductase. The RNA start site, as determined by S1 nuclease mapping and primer extention, is marked by an arrowhead. The positions of oligonucleotides referred to in the text are shown.

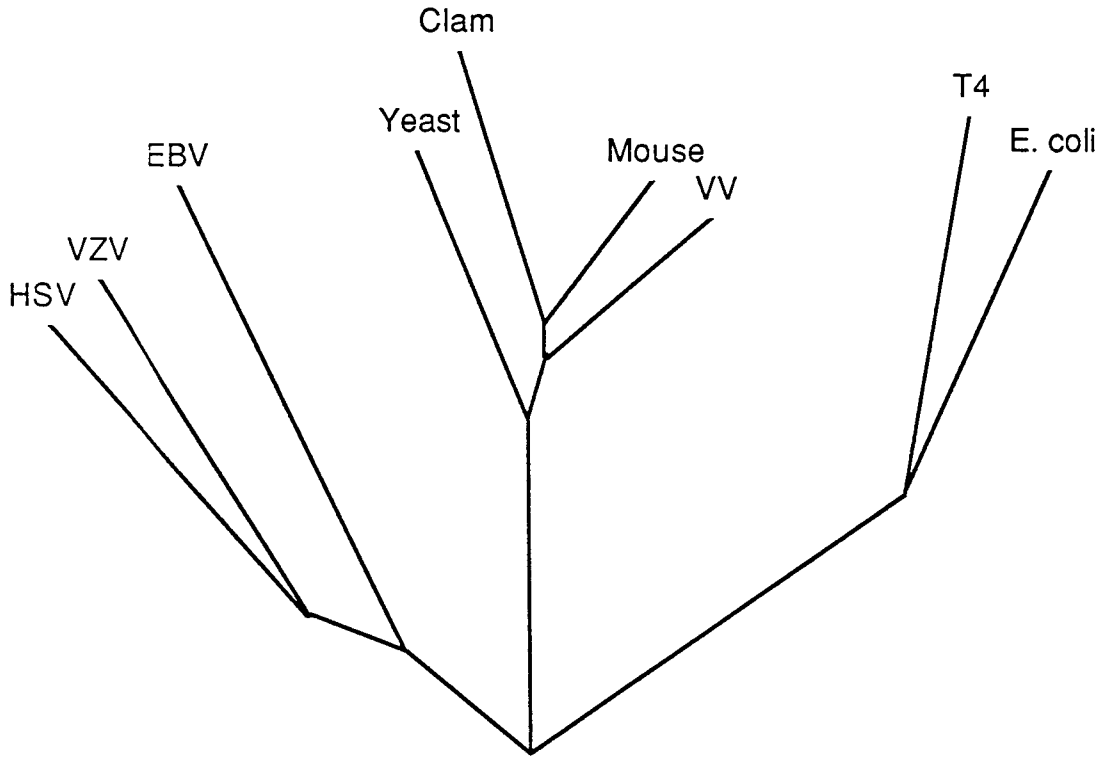


Figure II-5 Phylogenetic tree of nine R2 amino acid sequences generated by computer analysis (Slabaugh et al., 1988).

Chapter III
Evidence for Vaccinia Ribonucleotide Reductase Associating with Other Macromolecules

III.1 Introduction

After it had been determined that vaccinia virus has the genes and gene products for both subunits of the prototypical ribonucleotide reductase (Slabaugh et al., 1988; Tengelsen et al., 1989), my focus shifted to questions about macromolecular associations of RR. One rationale for using the vaccinia virus system to study deoxynucleotide metabolism is the unique localization of the virus during its intracellular lifecycle. The facts that the entire lifecycle occurs in the cytoplasm of infected cells, and that the virus codes for enzymes involved in both DNA precursor and DNA synthesis raise the question of how coordination of these interrelated processes occurs. One level of coordination is the complex feedback control of both RR and tk by deoxynucleotides. While it is conceivable that the modulation by deoxynucleotides can provide all the communication between precursor synthesis and DNA synthesis, it does not exclude other levels of regulation. One level of regulation I have investigated is that of macromolecular interactions. Other potential points of modulation are translational control and post-translational modifications.

Electron microscopic studies show the formation of inclusions in the cytoplasm of infected cells (Dales and Siminovitch, 1961). These large cytoplasmic aggregates have been described as "DNA factories" or "virosomes" (Cairn, 1960; Joklik and Becker, 1964; Dahl and Kates, 1970a). A rapidly sedimenting structure isolated at 3 hours post infection was shown to contain newly replicated viral DNA (Joklik and Becker, 1964). Dahl and Kates (1970a) demonstrated that these structures undergo alterations in density as a function of time during the infection cycle. A dense, rapidly sedimenting structure isolated 3.25 hours post-infection was determined to contain newly replicated viral DNA and was capable of synthesizing early and late viral mRNA *in vitro* (Dahl and Kates, 1970b). Treatment of the virosomes with pronase or detergent converted the rapidly sedimenting aggregate to DNA molecules which sediment similarly to mature vaccinia DNA molecules. In contrast, 98% of the virosome complex-associated DNA could be digested with DNase and the virosome complex sedimented as before. RNase treatment does not alter the rapid

sedimentation of the complex DNA. Two major early viral proteins, p34 and p40, are associated with virosomes and, to a lesser extent, other proteins. The amount of p34 sedimenting with the virosome is decreased 80% by DNase treatment while rapid sedimentation of p40 is relatively uneffected. The early work on kinetics of protein expression indicate that p34 is expressed shortly after infection, while p40 expression is coincidental with viral DNA synthesis (Polisky and Kates, 1975). There are contradicting data concerning the expression of p40 in the absence of viral DNA synthesis. Both proteins have been implicated as being packaged in the mature virion.

An *in vitro* analysis of the two major virosomal proteins showed that the complex could be dissociated by 2 M NaCl; however, the complex is restored upon removal of the salt by dialysis (Polisky and Kates, 1976). When more than 0.4 equivalents of exogenous DNA was added, the formation of the complex was greatly inhibited. At lower concentrations of added DNA a heterologous complex of vaccinia and other DNA could form, attesting that there is no sequence specificity required for DNA to be complexed. Denatured DNA proved to be 50% more efficient as an inhibitor of reconstitution. Approximately 40% of the complex-associated DNA was demonstrated to be single-stranded DNA. Two conclusions were drawn from these data: 1. the rapid sedimentation of vaccinia DNA complexes is dependent on a high stoichiometric ratio of complex-associated proteins, 2. protein-protein interactions are responsible for the complex formation.

While the two major virosomal proteins provide the bulk of the protein in the DNA-protein aggregates, there are several other proteins which cosediment with the virosome (Nowakowski et al. 1978 a, b; Polisky and Kates, 1972, 1975; Sarok and Joklik 1973). A topoisomerase activity was shown to sediment with the putative virosome at physiological salt concentrations but was absent when using low salt conditions in the gradient (Poddar and Bauer, 1986) . In addition, cytoplasmic extracts from infected cells revealed twenty-four "vaccinia DNA-binding proteins"(VDPs), ranging in molecular weight from 90 kDa to 12.5 kDa, retained on DNA-cellulose columns (Soloski et al., 1978). To date, none of these proteins had been assigned specific functions nor have the genes coding for them been identified.

A central question with which this thesis is concerned is: Is ribonuceotide reductase one of the proteins which is associated with the virosome *in vivo* ? Experiments I have conducted gave varying degrees of

evidence for the association of RR with a larger aggregate. Three different approaches were taken to probe directly for potential interactions. The first approach was an extension of the sedimentation studies used by Kates and others mentioned above. Briefly, infected cells were harvested early in infection, gently lysed, the nuclei spun out, and the extract loaded on a 20 to 50% sucrose gradient to isolate rapidly sedimenting viral DNA. The gradient was fractionated and then electrophoresed on an SDS-PAGE. After Western blot transfer, identification of RR was facilitated by polyclonal antibodies initially generated by Dr. Nancy Roseman. The second approach used bifunctional cross-linking agents which have been successfully used by others to demonstrate protein-protein interactions *in vivo*. Infected cells are incubated with a cross-linking agent, lysed, and immunoprecipitated with the anti-RR antibodies. Lastly, the same antibodies were used to perform immunocytolocalization of RR in infected cells. While not exhaustive the three methods represent divergent approaches that complement each other. One of the peculiarities of the mammalian RR, whose sequence is highly homologous with the vaccinia RR, is the relatively weak associations between the large and small subunits. At first glance, the lack of high affinity for these two subunits seems to be a complicating factor in probing for interactions of the enzyme with other macromolecules but provides an internal control for many of the experiments attempted.

III.2 Materials and Methods

Tissue Culture

Cells- Monkey kidney cell (BSC40) were used for most experiments and were grown to a 90-100% confluent monolayer in MEM/5% iron-supplemented calf serum/glutamine/P-S before being infected with partially purified viral stocks. Cells were split 1:10 and not used after15 passages.

Virus- Western Reserve stain (WR) was used for all experiments. All stocks were prepared from BSC_{40} cells infected at 0.1 pfu/cell and grown for two days at 37° C in a CO_2 incubator. Crude stocks were prepared by washing the infected monolayer with PBS, then scraping the cells off the plates in 10 ml PBS/10^7 cells, and afterwards spinning them in a clinical centrifuge at high speed for 5 minutes. The PBS was aspirated, then the pellet was resupended in 1 ml of MEM/5% iron supplemented calf serum/P-S/glutamine and frozen and thawed three times.

Preparation of Infected Cytoplasmic Extracts

The infected cell extract were prepared in a similar manner to Dahl and Kates (1975) except that the detergent was omitted in most extracts to maintain weak interactions. The cells were harvested 2.5 to 4.2 hours post-infection by washing the monolayer once in PBS, then scraping $2x10^7$ BSC_{40} cells into10 ml of PBS, then spinning the cells down in a clinical centrifuge for five minutes. The cells were resuspended in 1 ml of hypotonic lysis buffer (10 Tris pH= 8, 5 mM EDTA, 10 mM KCl, 10μM PMSF) and allowed to swell on ice for 10 minutes. The swollen cells were then placed in a 2 ml Dounce homogenizer (Kontes) and lysed with 12 strokes. For virosome prepared for sedimentation in 150 mM KCl, two lysis protocols were attempted: 1) Cells were resuspended in 0.5 ml of the 10 mM lysis buffer above and allowed to swell 10 min. The cells were placed in the homogenizer, the pestle lowered to the solution, 0.5 ml of 300 ml lysis buffer was placed on top of the plunger, and the cells immediately lysed with 12 strokes of the homogenizer 2) 0.5 % final concentration NP-40 was added to the lysis buffer which was 150 mM KCl. 1 mM $MgCl_2$ (no EDTA present in this case), dATP, 1 mM ATP, 1 μM dATP, and 200 μM CDP were

added to lysis buffers and gradients as indicated. The resulting cell extracts were centrifuged for 4 minutes at 800xg to pellet the nuclei.

Sucrose Gradients and Fractionation

The crude virosome extract was loaded on top of 10-ml 20-50% sucrose gradients, each with a 80% sucrose cushion. Identical buffer conditions as final cytoplasmic extract were used in the gradients except detergent was left out of all the gradients. The gradients were centrifuged for 80 minutes at 18,000 rpm in a SW 41 Beckman rotor at 4° C. 1.2-ml fractions were collected from the bottom with an ISCO fractionator and diluted 1:1 in 2x SDS-PAGE loading buffer. In later experiments fractions were either concentrated by TCA precipitation with 5 µg/ml of BSA added, or dialyzed against 0.5% NH_4HCO_3, 5 mM EDTA, 40 µg/ml PMSF, lyophilized 12 hours and then resuspended in 100 µl 2X SDS-PAGE loading buffer.

Electrophoresis and Western Blotting

SDS-PAGE was used to resolve proteins from the fractions of the sucrose gradients. The proteins were then transferred onto Immobilon-P membrane (Millipore) at 200 mAmps. overnight in an Idea Scientific semi-dry transfer apparatus.

Antibodies and Detection

Rabbit polyclonal antibodies were generated to separate fusion constructs with R1 and R2 polypeptides fused to trpE by Dr. Nancy Roseman (Howell et al., manuscript in preparation). The antibodies used in this study were specific to the C-terminal 30 kDa of R2 (94-30) and the C-terminal 27 kDa of R1(69-27), and were used at 1:1000 and 1:500 dilutions respectively. The blots were blocked with 1% gelatin in TBST (20 mM Tris, 140 mM NaCl, 1 mM EDTA, 0.1% Tween 20) for 45 minutes and then incubated with the primary antibody diluted in TBST for one hour. After three ten-minute washes with TBST the blots were then incubated for 45 minutes with a 1:7,000 dilution of (Promega) goat anti-rabbit IgG conjugated to alkaline phosphatase. The blots were washed 3 times with TBST and once with Dev buffer (0.1 M ethanolamine,

50 mM $MgCl_2$, 100 mM NaCl pH=9.6) before adding 44 µl NBT and 33 µl BCIP (BRL) in 10 ml Dev buffer to develop the blots for 30-120 minutes. The reaction was quenched by rinsing in TBST twice, in water once, and air drying the blots.

Labeling Infected Cells and Cross-Linking

For each cross-linker used a series of dilutions was added to infected BSC_{40} cells which had been labeled with 100 µCi/ml of [^{35}S]-methionine at 2-4 hours post-infection. Typically cells were washed 2 times with PBS and placed on ice. DSS (Pierce) and DSP (Pierce) were dissolved in DMSO at a concentration of 100 mM and diluted into PBS with 1% DMSO. Sulfo-SADPH (Pierce) was made directly in PBS at final concentration used and added to cells in dim room with red light source. The cells were washed 2X in PBS with 50 mM ammonium acetate to quench and remove excess cross-linker. For the sulfo-SADPH crosslinking, 2ml of PBS added to the plates and a UV transilluminator was placed over the open plates for 8 minutes After removing the PBS 1 ml of RIPA buffer with 50 mM ammonium acetate was added to each plate and the plates rocked occasionally on ice for 30 minutes. The resultant lysate was placed in Eppendorf tubes and centrifuged at 15,000 rpm at 4° C for ten minutes. The supernatant was moved to a new tube and incubated on ice for 30 minutes. 5 µl of anti-RR antibodies were added to the extracts and incubated on ice for 60 minutes. 100 µl of a 10% slurry of protein A-Sepharose beads (Sigma) in RIPA was added to each tube and the contents rotated at 4° C for 60 minutes. The beads were spun down in the cold and washed with 1 ml of RIPA three times. 40 µl of 2X SDS-PAGE loading buffer was added to each tube and then place in a boiling water bath for 5 minutes. Alternatively, the cross-linked partner was released from the protein A-antibody-RR complex by incubating in 100 mM DTT for 30 minutes at 37°C and the resultant supernatant removed to a new tube. 2X SDS-PAGE loading buffer was then added to both the supernatant and the beads and heated as above.

SDS-PAGE and Fluorography

The gel system was the same as in the previous section except gradient gels were used as noted in figures. After electophoresis the gel was prepared for fluorography by rocking in 50% methanol/10% acetic acid for 30 minutes

followed by another soaking in 5% methanol/7% acetic acid for 30 minutes. After removing fixers by a 30 minute soaking in distilled water the gel was saturated with salicylate by soaking in a 1 M solution of sodium salicylate and then dried in a vacuum gel drier. The gel was placed on x-ray film for two days before development.

Immunolocalization

BSC_{40} cells were split 1:5 two days before the experiment and added to 12-well plates with 18-mm glass coverslips in the wells. When the monolayer on the coverslip was 80-90% confluent the cells were infected with 2 PFU's/cell of a partially purified vaccinia virus stock in a volume of 333 µl/well. The inoculum was removed after one hour and 1 ml of MEM complete was added. At 3 hours post-infection 1 µl of BrdUrd labeling mix (Amersham) was added. At 3.4 hours post-infection the cells were washed two times with PBS and fixed.

The cells were fixed by adding 3 ml of freshly prepared 4% paraformaldehye in 1x PBS and incubating for 10 minutes at room temperature. The wells were washed 2x with 2 ml/well of PBS and then 3 ml/well of 100% methanol was added to permeabilize the cells. After 2 minutes the methanol was aspirated and the wells rinsed 2 times with PBS. The second rinse was left for 30 minutes to rehydrate the cells on the coverslips. At this point the coverslips were removed and placed on a sheet of parafilm in a humidity chamber, and 100 µl of 3% BSA in PBS was layered on each as a blocking agent. All dilutions of antibodies were done in 3% BSA in PBS except the anti-BrUdR, which was used as purchased from Amersham.

Initial results using dilutions of the antibodies ranging from 1:10 to 1:500 showed only a decrease in the total signal with no improvement of apparent detection of the RR in infected cells in relationship to the pre-immune sera. The immune serum was diluted 1:5 in PBS and 2 ml was serially passaged over five 100-millimeter tissue culture plates of uninfected cells which had been fixed in the same manner as above. In addition, the infected and fixed cells were trypsinized with 0.01 mg/ml trypsin (Difco) for 8 minutes at room temperature to reexpose buried epitopes (Harlow and Lane, 1988).

The anti-R1 antibody (69-27) was used due to the lower-signal-to noise ratio. The optimal conditions were as follows: 69-27 was diluted 1:20 in 3% BSA/PBS and incubated overnight at 4 °C. The coverslips were then washed 3

times over a twenty-minute period and 60 µl of 1:400 dilution of biotinlyated goat anti-rabbit (Pierce) was added. The coverslips were then incubated in a humidity chamber for 30 minutes at room temperature. After three rinses with PBS, 60 µl of a 1:100 dilution of streptavidin-FITC (Pierce) was added and the coverslips incubated for 20 minutes. Localization of newly synthesized DNA was determined as recommended by Amersham using an anti-mouse antibody conjugated to Texas Red dye (Vecta) at 1:100 dilution. The BrdUrd antibody system was layered on next to minimize any artifactual localization of the anti-RR system. After the final wash the coverslips were removed from the parafilm and mounted on microscope slides with 10 µl DAPCO (Sigma).

The slides were viewed on a Zeiss microscope equiped with fluorescent optics and filters for FITC and rhodamine. Pictures were taken with Ektacolor (ASA 1000) Kodak film with one-minute exposures.

III.3 Results

Sucrose Gradient Experiments

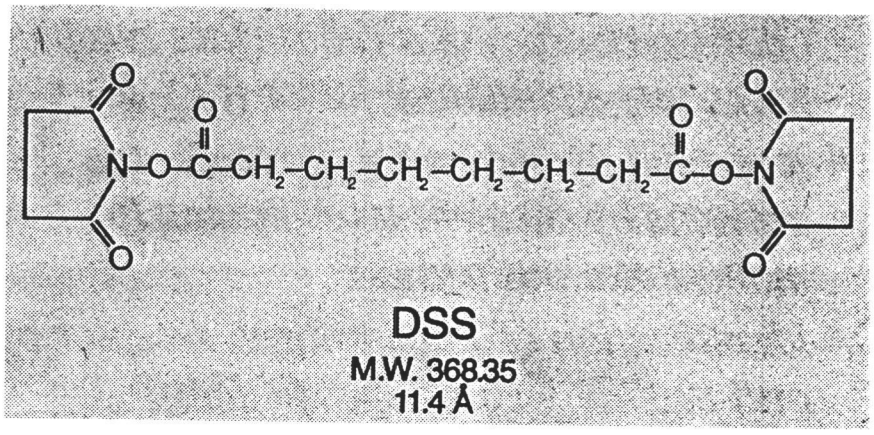
Previous experiments done in this lab have shown a small amount of RR activity sediments with the virosome pellet using differential sedimentation (Slabaugh et al., 1984). In contrast, DNA polymerase activity stays much more tightly associated with the virosome fraction. To see if the RR that is associated with the virosome is specific and not just part of the supernatant trapped in the pellet I have utilized immunological probes, to follow the RR through sucrose gradients after an initial low speed centrifugation to pellet the nuclei. The use of Western blots to analyse the sedimentation through the sucrose gradients circumvented the difficulty of trying to maintain enzyme activity while dialysing dilute enzyme to remove inhibitory concentrations of sucrose and salt. Two concentrations of salt were initially tried with vaccinia infected cells. Labeling with [^{3}H]-thymidine allowed the sedimenting viral DNA.to be followed. While there was a wide variance in the profile of the labeled DNA, Figure III-1 is representative of the results, which were similar to those previously reported in the literature. An uninfected control demonstrated that this rapidly sedimenting complex is specific for vaccinia infected cells. After establishing the profile of viral DNA sedimentation in the system I used, Western blots were done on fractions of the gradient. In general, there was no evidence of sedimentation of the RR with the virosome (Figure III-2). Since allosteric effectors of RR can affect the quaternary structure of enzyme (Brown and Reichard, 1969 a,b), lysis and sedimentations were done in the presence of physiological concentrations of effectors (1 mM ATP, 1 μM dATP, 1 mM Mg^{2+}) and a substrate (200 μM CDP). The initial attempt demonstrated that a small fraction of the R2 subunit protein could be held with a rapidly sedimenting aggregate and 150 mM NaCl decreased the amount of cross-reacting host protein relative to the 10 mM NaCl conditions (Figure III-3). The experiment was repeated with dilutions of the cytoplasmic extract to quantitate the relative proportion of RR sedimenting. In addition the blot was cut and the top half probed with anti-R1 antiserum and the bottom with anti-R2 antiserum to determine if both subunits were sedimenting with the virosome. From the dilutions of the cytoplasmic extracts the amount of R2 sedimenting was estimated to be 2-4%. Ironically, the putative host R1 sedimented with the virosome while there was no evidence of the viral R1

sedimenting with the virosome under these conditions. Further experiments established that CDP was not essential to maintain the small amount of rapidly sedimenting viral R2 while ATP and to a lesser extent dATP were essential.

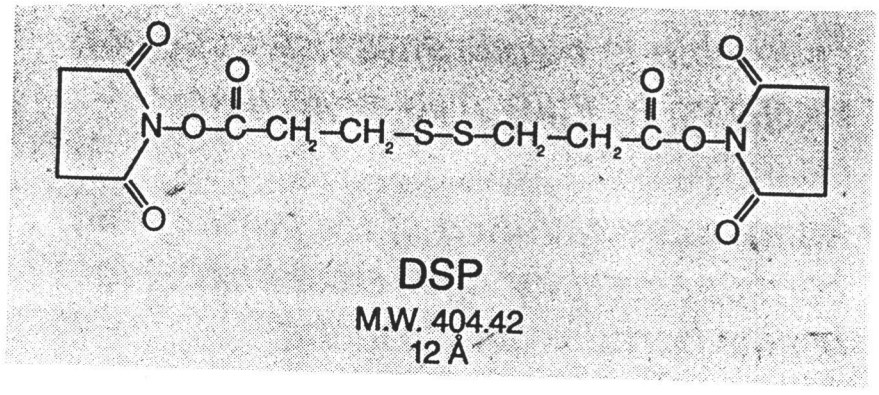
Chemical Cross-Linking *In Vivo*

Several groups have used cross-linking reagent to demonstrate interactions between proteins *in vivo* (Halpin et al., 1981; Chelsky and Dahlquist, 1980; Zarling et al., 1982; De Gunzburg et al., 1989) . The use of polyclonal antibodies to a given protein allows the investigator to pull out the unknown partner which is cross-linked. The work done on this project centered on the use of three cross-linking agents:

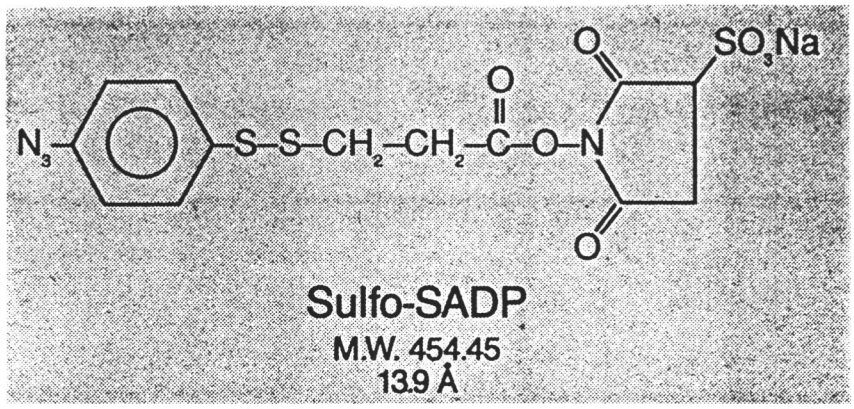
1. Disuccinimidyl suberate (DSS) is a non-cleavable amine-reactive homofunctional cross-linker:



2. Dithio*bis* (succinimidylpropionate) (DSP) is a cleavable analogue of DSS:



3. Sulfosuccinimidyl(4-azidophenyldithio)propionate (sulfo-SADPH) - a photoactivatable amine-reactive heterobifunctional cleavable cross-linker:



Early experiments with DSS pointed out the importance of quenching the cross-linker after the *in vivo* incubation. If the cross-linker is not quenched, cross-linking to the antibody system used to immunoprecipitate can occur and lead to a high-molecular-weight antigen-antibody aggregate. In addition, without being able to cleave the cross-linked product, the identification of the protein(s) which is being cross-linked to the RR is difficult. For these reasons two modifications to the early work done with the cross-linking reagent DSS were incorporated into subsequent experiments:

A. After the cross-linking incubation period, all washes of the cells and lysis buffers included a quenching agent (50 mM ammonium acetate in most cases). The cell extracts were allowed to incubate at least 30 minutes on ice before the addition of the primary antibody (see Figure III-5).

B. Cleavable cross-linkers were used so that the cross-linked species could be separated after immunoprecipitations.

Increasing amounts of either DSP or SADPH resulted in a decrease in the amount of RR running at the correct molecular weight on SDS-PAGE (Figure III-6). Cleavage of the cross-linked immunoprecipitate yielded only the original RR band and no new radiolabeled species. While it is tempting to conclude that there are not interactions with other proteins *in vivo* , the inability to cross-link the R1 and R2 polypeptides together argues against interpreting

these results as broadly as this. There also remains the recovery of the original immunoprecipitated species when the cross-linker is cleaved. Since the system is quenched before the antibodies are added, cross-linking to the antibody system *in vitro* is not likely. At this point I think a combination of three possibilities exist:

1. A general non-specific cross-linking to host proteins occurs over the incubation period as seen on gels run with total proteins (see Figure III-7).
2. There is a specific host protein(s) which is cross-linking to the RR and is not labeled significantly with the labeling protocol used.
3. These proteins, especially R1, form higher molecular weight homoaggregates *in vivo.* There is evidence for this in vitro from the *E. coli* RR when dATP binds to the specificity site (Reichard and Brown, 1969).

Immunocytolocalization of RR

The sucrose gradient experiments gave indications that RR may be associated with the virosome and newly replicating DNA in vaccinia-infected cells. In order to determine if RR is localized at the sites of vaccinia DNA replication immunolocalization experiments were performed. Initial experiments focused on the use of only the crude antiserum against the R1 (N.A.R. 69-27, bleed 7). This antibody appeared to give the lowest background in uninfected cells. There was a consistent problem with probing VV infected cells for RR: When BSC_{40} cells are infected at high multiplicities of infection (moi=15-20), the cells round up and are tenuously attached to the glass disks used for these studies. HeLa cells were not much better in this respect. Loss of cells (95%) and a high non-specific background seen with the pre-immune sera resulted from the cytopathic effects seen with high moi infections. In order to circumvent these problems, low moi infections were attempted but very little difference between pre-immune controls and immune sera could be seen for a wide range of dilutions (1:10 down to 1:500). A wide variety of alterations in fixing, blocking, and washing were attempted to increase the signal-to-noise ratio. Ultimately a series of conditions were devised which attenuated this problem and demonstrated a localization of the R1 polypeptide to the viral inclusion bodies in several independent experiments (Figure 3-8). A pre-

immune control which was not preabsorbed gave no indication of immunolocalization of R1 to the virosomes.

III.4 Discussion

The three techniques used have given varying degrees of evidence for RR being involved in higher levels of macromolecular organization. Each line of evidence has its weakness, but *in toto* the data suggest that there are interactions that go beyond the purely enzymatic role of RR in vaccinia virus replication. A great deal of preliminary work was done to optimize the conditions used and in many cases yielded equivocal results. The sucrose gradient experiments were very sensitive to the lysis conditions used (volume the cells were homogenized in, composition of the buffer used and presence of effectors of RR). The small amount of RR seen sedimenting with the newly replicated DNA suggests that the interactions are weak and/or dynamic *in vivo* and may be dependent on multiple interactions.

Although the cross-linking experiments results alone are inconclusive, taken in conjuction with recent results from anti-idiotypic antibodies (see Chapter 4), the potential interaction with a host protein remains an attractive area to study. A host 40-kDa protein has been detected by the anti-idiotypic antibody approach (current work) and may possibly be involved in host-viral interactions involving RR and viral DNA synthesis. Given these results it would be worthwhile to explore cross-linking and probing with the newly generated anti-anti R1. The other possibility of *in vivo* aggregation of the RR is worth exploring in the context of understanding quaternary structure *in vivo* . This may lead to insights into why the turnover number for RR is so low *in vitro.*

The experiments on immunolocalization of the R1 subunits gave the most definitive indication of the RR being associated with newly replicating DNA. The ability to pulse-label newly synthesized DNA with BrdUrd and detection of the replicated DNA with a monoclonal antibody to BrdUrd has provided a powerful tool to probe the localized viral replication centers unambiguously. Immunocytolocalization has proved to be not trivial when probing for RR in the vaccinia system. By increasing the m.o.i., the investigator can increase the level of the viral enzyme 10-20 fold (Slabaugh et al., 1984) above the host background, but at the price of decreased signal-to-noise ratio when using a polyclonal antibody. In addition, the infected cells display such severe cytopathology that fixing, multiple incubations, and washings leave few cells and the cells left display poorly defined morphology. Decreasing the m.o.i.

does help retain the cell morphology but decreases the target antigen and also delays the onset of DNA synthesis.

Using a fixation procedure that cross-links soluble antigens before permeabilization with organic solvents has proven to give the best results in terms of preserving the cell morphology as well as indicating localization of R1 to the virosome. Other groups have found that fixation procedures can have profound effects on the localization of a given antigen. In addition, I utilized a brief proteolysis of the fixed cell as recommended by Antibodies manual (Harlow and Lane, 1988). This treatment, in theory, is supposed to reexpose antigen sites masked and buried by the cross-linking procedure. Unfortunately this also removed a substantial number of tenuously anchored cells so the incubation time and concentration of trypsin which produced satisfactory results was critical. The procedure which had to be used to demonstrate localization of RR suggests that these associations may involve several layers of loosely associated proteins. The associations apparently do not survive organic fixing techniques. The necessity of proteolysis to expose the virosome associated RR may indicate other proteins masking the RR.

In light of the results of the next chapter, some of these techniques could be pursued again with a reasonable possibility of success. Refinements for the cell fractionation experiments could include using macromolecular excluders such as polyethylene glycol. Also, the use of other cross-linking reagents would allow different geometries of interactions to be detected. Permeabilizing cells before applying cross-linking reagents would allow the cross-linker to diffuse to the cytoplasmic space, allowing the use of a broader spectrum of cross-linkers at lower concentrations. The combination of cleavable cross-linkers and sedimentation of the virosome is an avenue that has the potential of revealing specific pairs of interactions by running two dimensional gels on the cross-linked products. Affinity purifying the antibodies, now that sufficient quantities of the recombinant RR polypeptides are available to be immobilized, has the potential of decreasing the non-specific background seen. Using a cell line which does not display the severe cytopathic effects of the lines we had available at the time could make the immunocytolocalization of deoxynucleotide metabolizing enzymes of vaccinia virus a more clear cut experiment. Overexpression of a given polypeptide in vaccinia virus infected cells is now routinely possible with high efficiency transfection and plasmid expression systems developed in the last couple of years, although I have tried to use the

endogenous system in case there are a saturable number of interaction sites. While these results suggest possible associations and the need for further experimention and refinement of the conditions used, the identification of specific proteins involved in these putative interactions is of paramount importance to demonstrate specificity. While chemical cross-linking was one avenue attempted, the generation of anti-idiotypic antibodies indicated a specific interaction first and hence was pursued at this time.

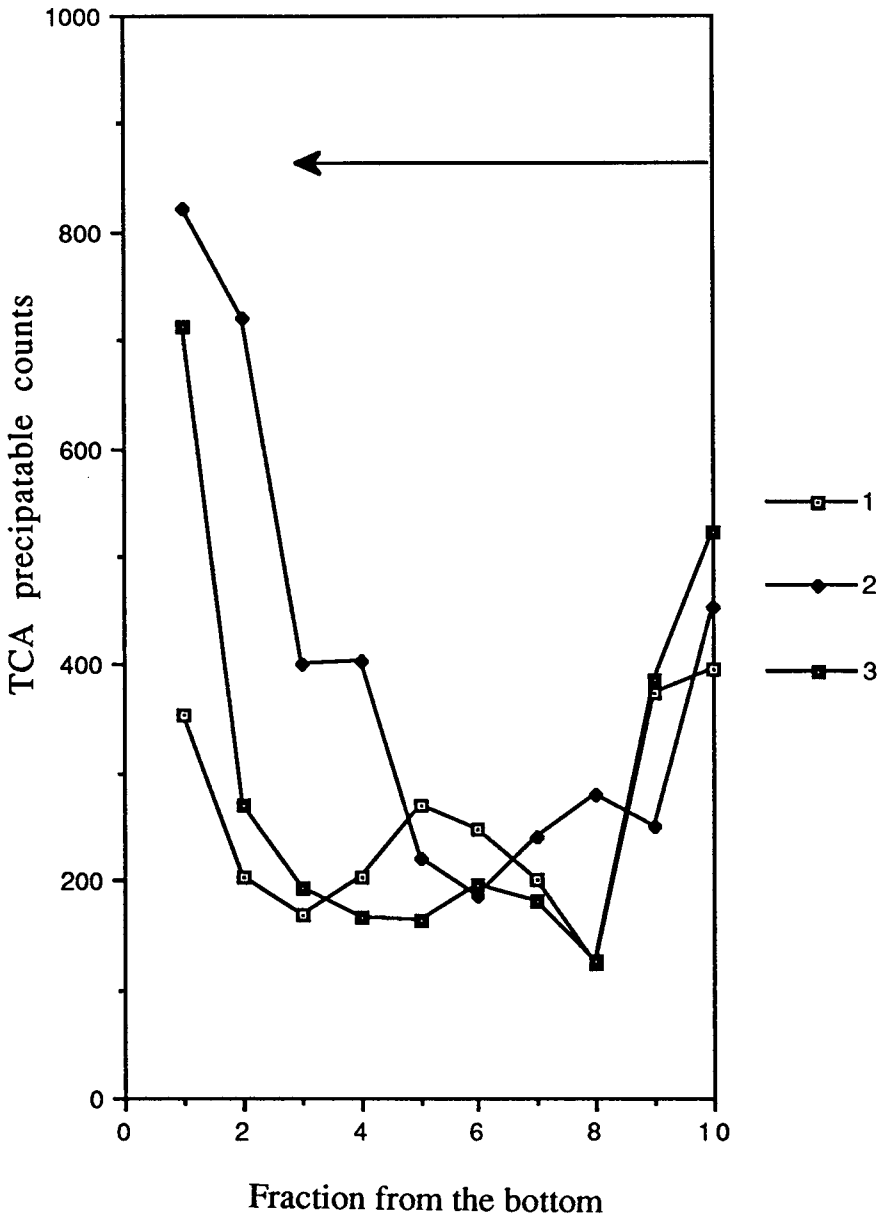


Figure III-1 Rapidly sedimenting vaccinia virus DNA. Vaccinia-infected BSC_{40} cells were labeled at 2-3.5 hours with [^{3}H]thymidine and prepared as per Dahl and Kates (1975). 100 µl of each 1.2 ml fraction was TCA precipitated and counted. 1 infected cells were lysed and extracts run on gradient in 10 mM KCl buffer; 2 infected cells were lysed in the presence of 150 mM KCl buffer and NP-40; 3 infected cells were swollen in 10 mM KCl and homogenized in 155 mM KCl final concentration buffer. All gradients were run without detergent.

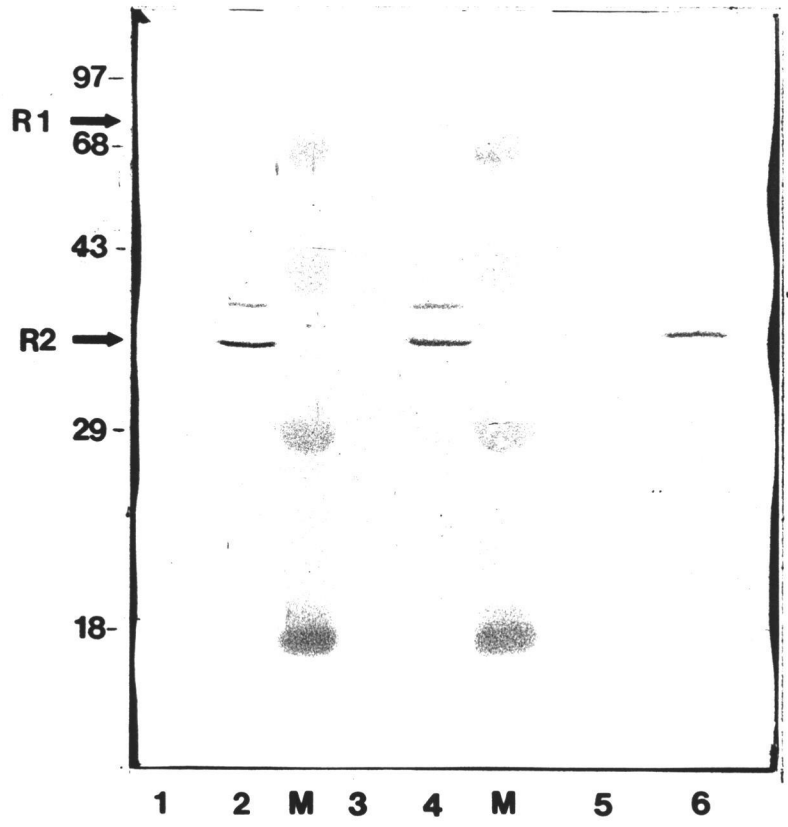


Figure III-2 Western blot probing for ribonucleotide reductase sedimenting with the virosome using different salt and lysis conditions. 20 μl of the top and bottom fraction of the 20 to 50% gradient is loaded onto the gel. Blot was probed with both anti-R1 and anti-R2. Odd numbered lanes are the bottom of the gradients. Even numbered lanes are the top. Lanes 1 and 2 were cells swollen in hypotonic buffer and lysed and sedimented in the presence of 155 mM KCl. Lanes 3 and 4 were cells lysed in 150 mM KCl and 0.5% NP-40 and sedimented in 150 mM KCl buffered sucrose gradient. Lanes 5 and 6 were cells lysed in hypotonic buffer (10 mM KCl) and sedimented in 150 mM KCl buffered sucrose gradient. (12.5% SDS-PAGE)

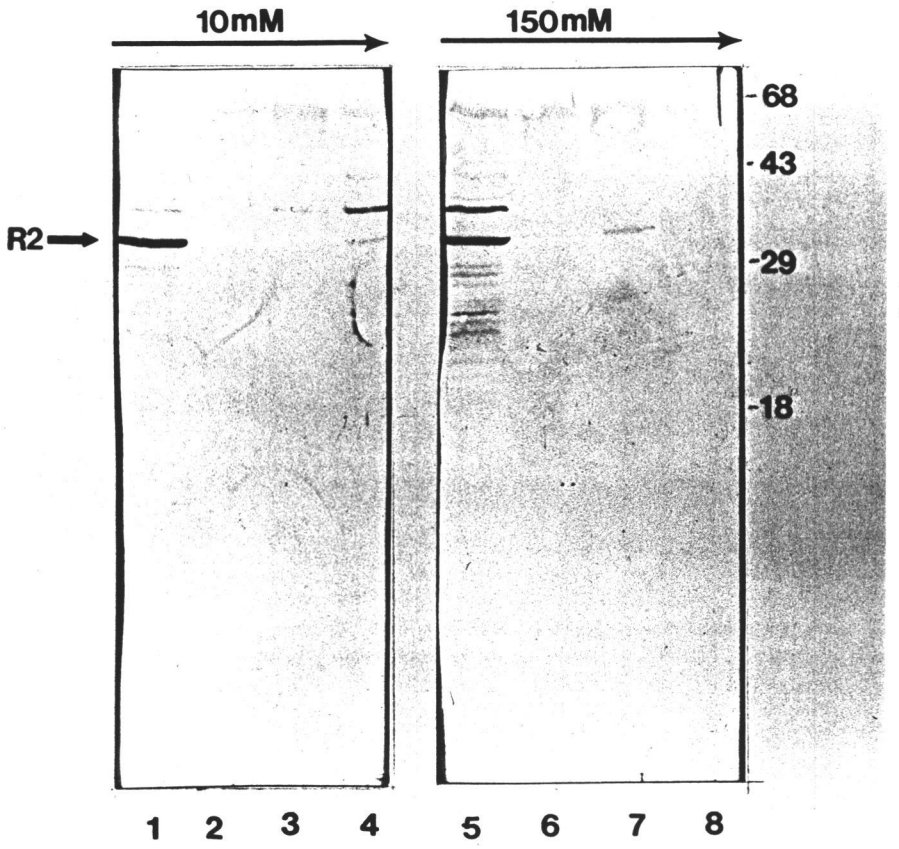


Figure III-3 Western blot probing for R2 comparing low salt to physiological salt sedimentations. Lane 1-4 the virosome was sedimented in 10mM KCl buffer. Lane 5-8 the virosome was sedimented in 150 mM KCl buffer. Lanes 1 and 5 correspond to the bottom of the gradient, with lanes 4 and 8 corresponding to the top of the respective 20 to 50% sucrose gradients. Lysis and sedimentations were done in the presence of Mg^{2+}, ATP, CDP, and dATP. Arrows indicate direction of sedimentation. (15% SDS-PAGE)

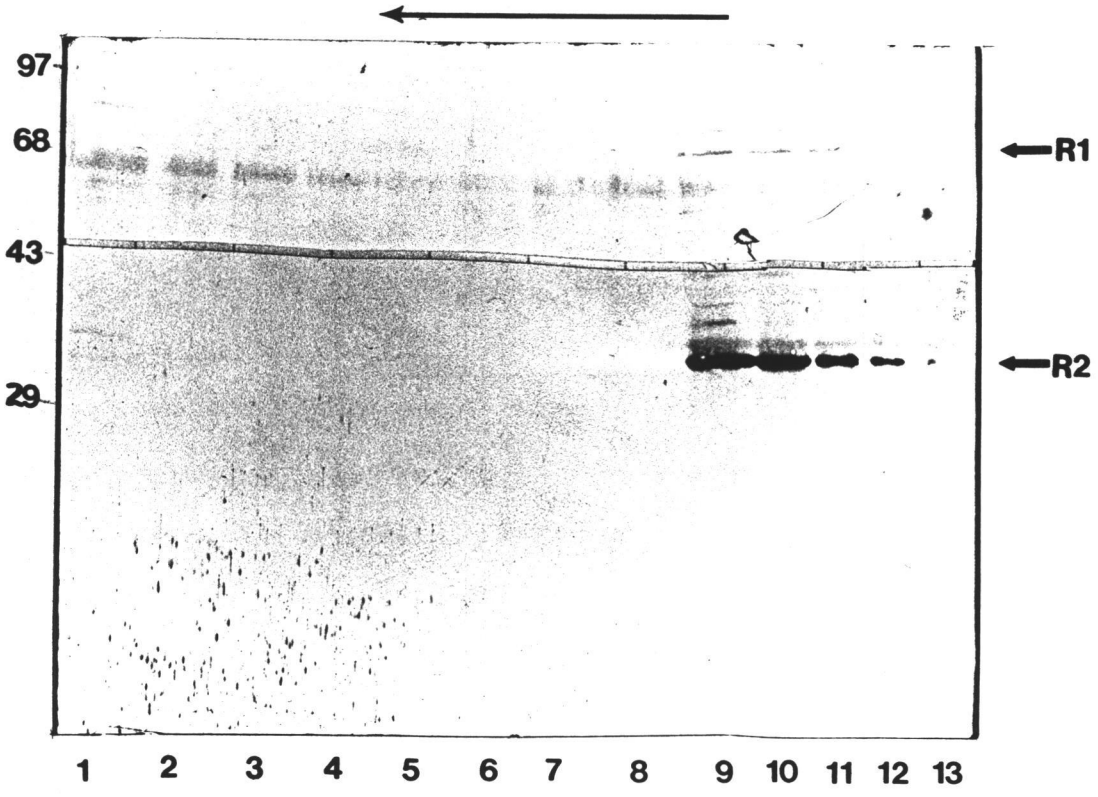


Figure III-4 Relative levels of RR subunits sedimenting as estimated by Western blot. A 20 to 50% sucrose gradient was run on vaccinia virus infected BSC40 cell extracts harvested at 4.2 hours post-infection. Final concentrations in lysis buffer and gradient were 1mM $MgCl_2$,1 mM ATP, 200 μM dATP, and 1 μM dATP. Lane 1 is the bottom of the gradient and Lane 9 is top. Lane 10-13 are 1:2 serial dilutions of lane 9. One-quarter of each 1.2-ml fraction is loaded in each lane. (12.5% SDS-PAGE)

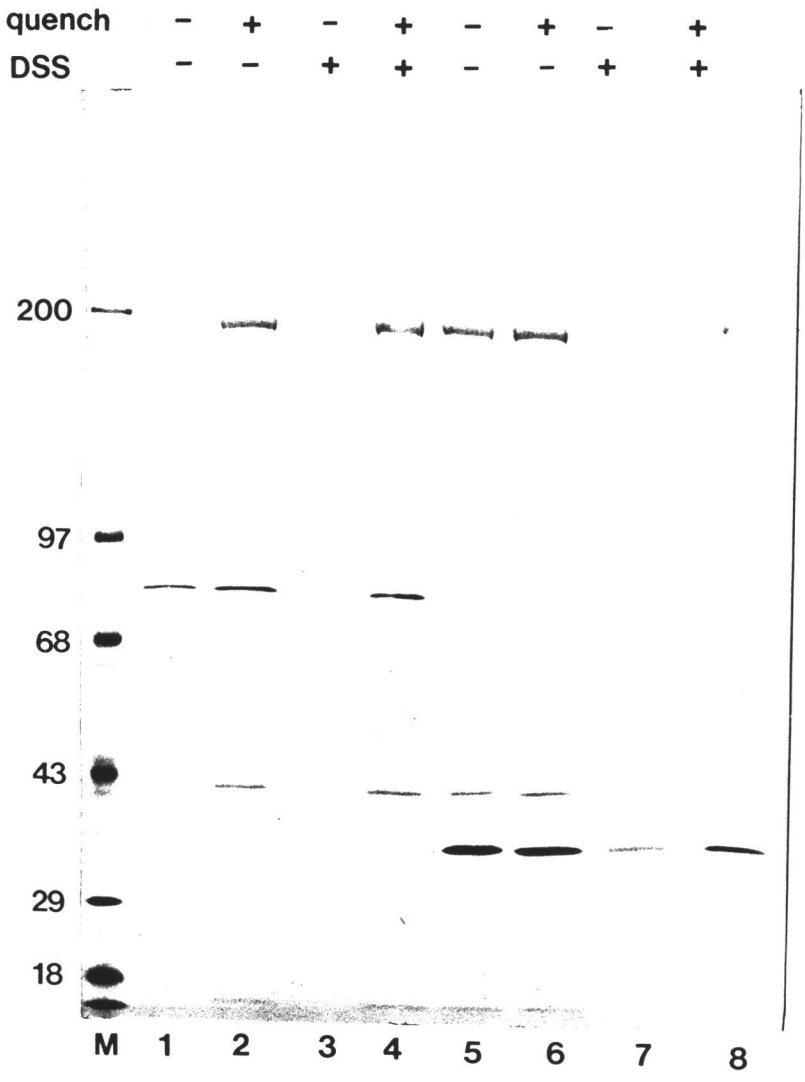


Figure III-5 *In vivo* cross-linking of vaccinia RR with DSS and quenching. Lanes 1-4 are immunoprecipitated with anti-R1. Lane 5-8 are immunoprecipitated with anti-R2. The addition of 250 μM DSS and/or 50 mM ammonium acetate is noted above the lanes. (5 to12% gradient SDS-PAGE)

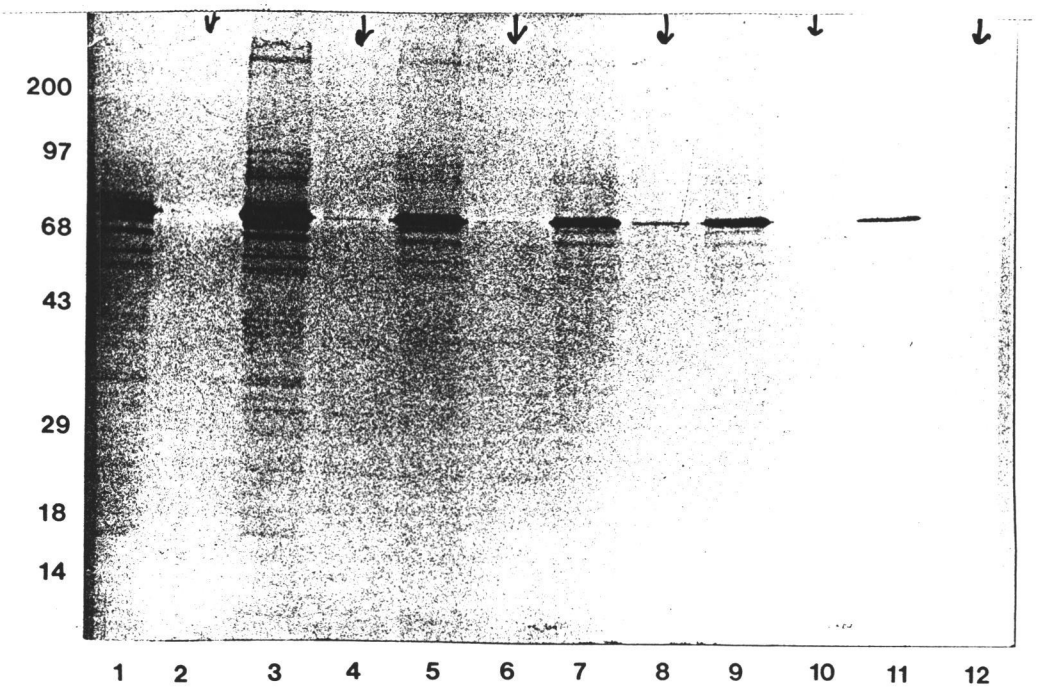


Figure III-6 R1 cross-linked with sulfo-SADPH *in vivo.* Vaccinia-infected cells were incubated as described in Materials and Methods with concentrations of sulfo-SADPH indicated below gel. Lane 1 and 2 are controls with no UV light treatment. Odd-numbered lanes are immunoprecipated proteins boiled in SDS-loading buffer after cross-linker is cleaved and cross-linked species removed. Even-numbered lanes (arrows in lanes) are soluble extracts released from immunoprecipitated R1 with 100 mM DTT and then boiled in SDS-loading buffer. (10 to 20% gradient SDS-PAGE)

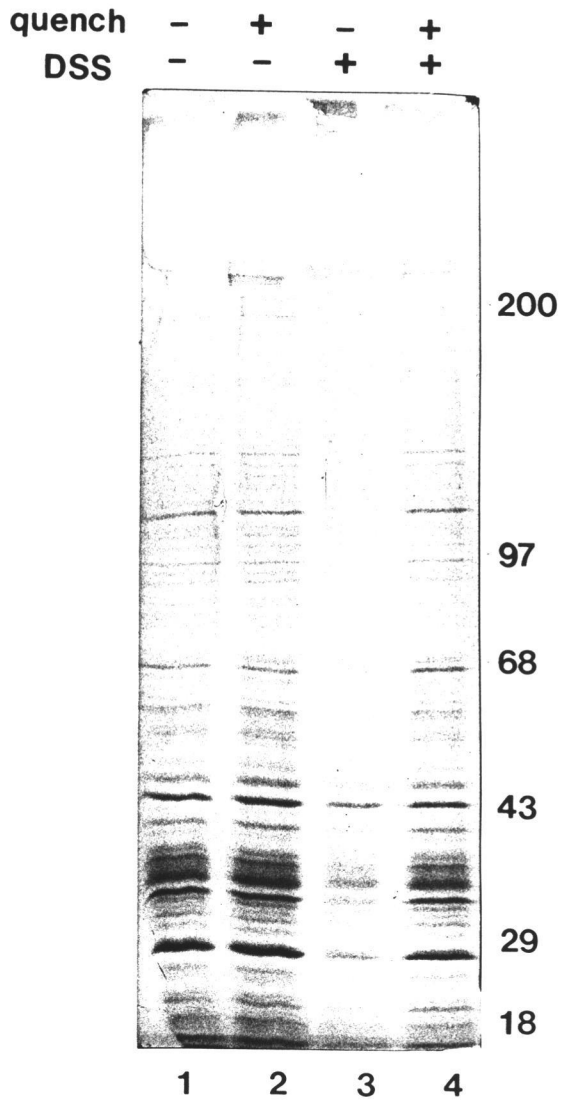
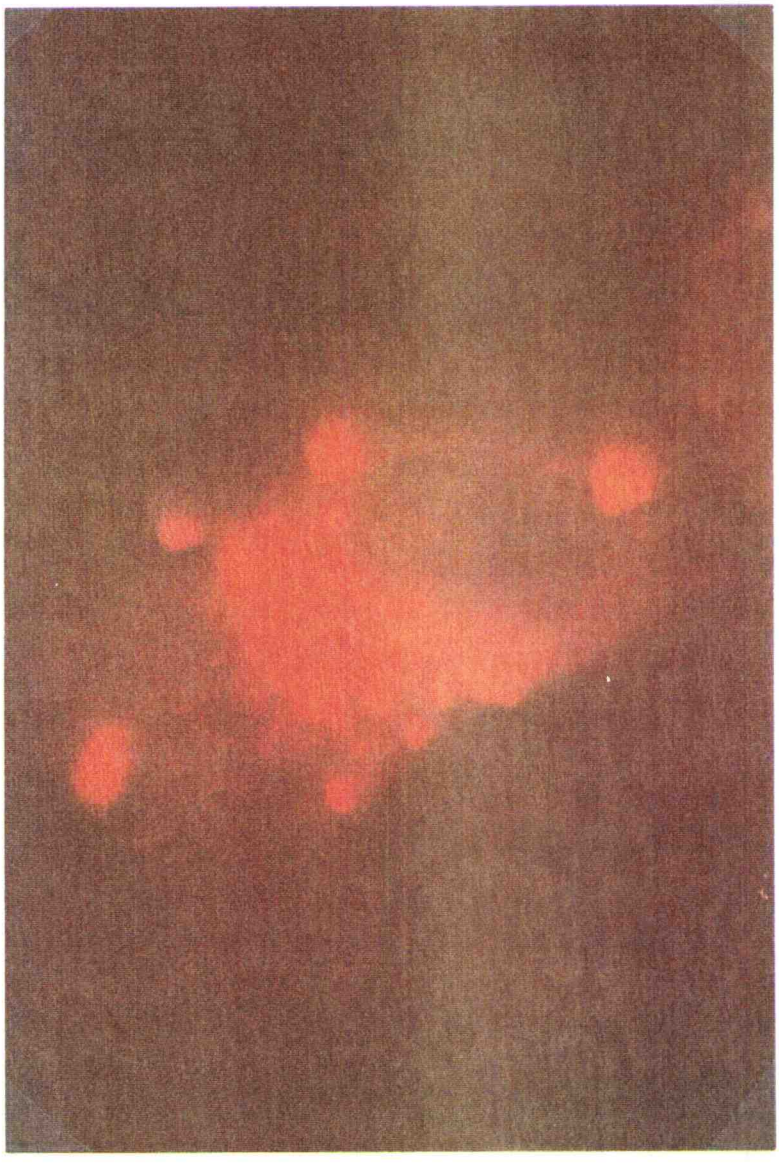
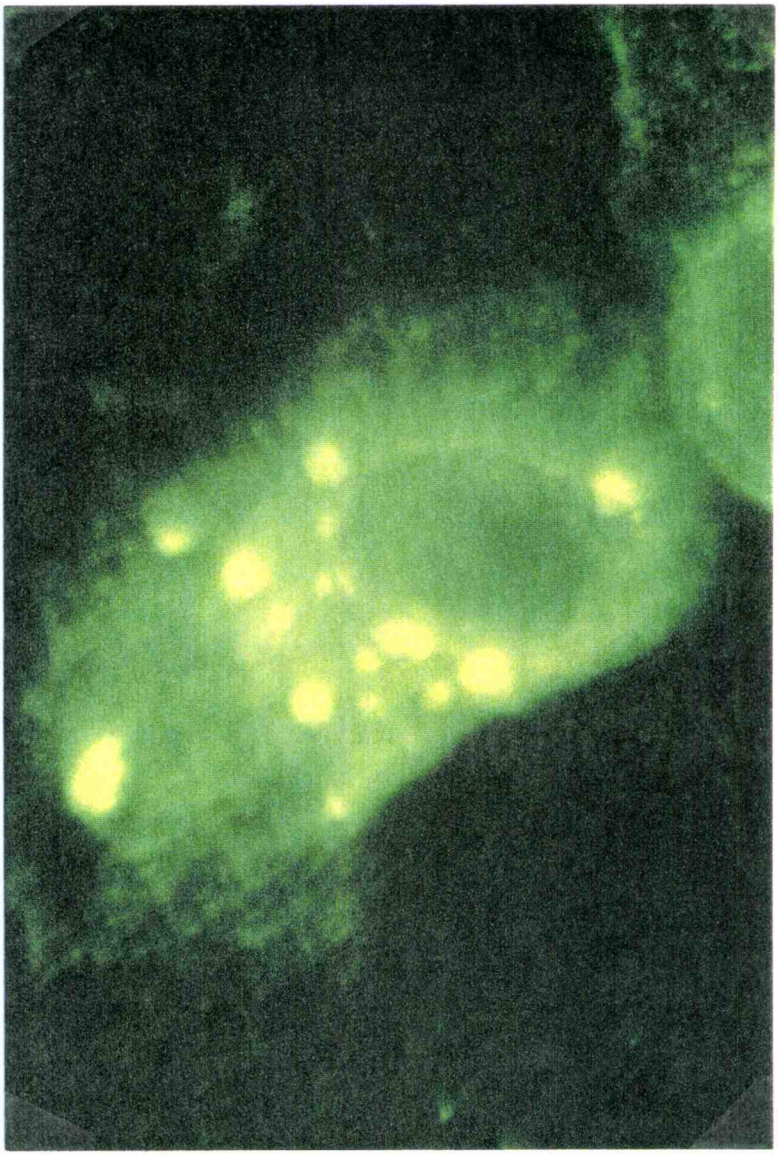


Figure III-7 *In vivo* DSS cross-linked whole extracts. 10 µl of supernatant of R1 immunoprecipitated extracts from Figure III-5 were run on a 5 to 12% SDS-PAGE.



(A)

Figure III-8 Immunolocalization of R1 to viral inclusion bodies.
Vaccinia-virus infected BSC_{40} cells (M.O.I. = 2 pfu), labeled at 3.5 hours post-infection with BrdUrd and fixed at 4 hours post-infection. The cells were incubated with anti-BrdUrd and anti-R1 as described in Materials and Methods. (A) Immunolocalization of newly synthesized DNA (filter set for rhodamine) (B) Immunolocalization of R1 (filter set for FITC)



(B)

Figure III-8 continued

Chapter IV
Acidic Carboxy Terminus of Vaccinia Virus DNA Binding Protein Interacts with Ribonucleotide Reductase

IV.1 Introduction

Probing levels of macromolecular organization, especially of proteins involved in sequential steps of a given pathway or biochemical process such as DNA replication, can reveal subtle yet important interactions. A fundamental question is whether there is more than a temporal linkage between DNA precursor metabolism and DNA synthesis. Many interactions that are significant *in vivo* are such that isolation of a complex of proteins *in vitro* is impossible because of the transient nature of the association or the weak interactions, which become destabilized upon dilution accompanying any isolation or extraction procedure. There are methods for approaching these questions, such as chemical crosslinking agents, immunocytolocalization, and partitioning enzymes with polyethylene glycol. We have employed some of these techniques and have obtained some evidence for localization of ribonucleotide reductase at sites where viral DNA synthesis occurs. Another approach has been the use of anti-idiotypic antibodies for detecting unknown interactions (for review see Venter et al., 1984). The most common application of anti-idiotypic antibodies has been in the receptor-ligand field (Gaulton and Green, 1986). By generating an antibody to a ligand and then generating antibodies to the antibody, in theory some of the second round antibodies will mimic the binding surface of the ligand and hence bind to the unknown receptor. An extension of this idea has been utilized to probe for protein-protein interactions (Figure IV-1) both in our laboratory (Young and Mathews, 1992) and others (Vaux et al., 1990). This technique has allowed us to (1) detect a previously unknown interaction; (2) conclusively localize the relevant protein in infected cells; (3) unambiguously identify the viral gene encoding for this protein; and (4) hypothesize a testable function for this protein.

Over the last few years there has been increasing evidence for association of enzymes involved in deoxyribonucleotide biosynthesis in T4 bacteriophage-infected *E. coli* (Chiu and Greenberg, 1973) Orthopoxvirus-infected eucaryotic cells provided an attractive analogous system for probing

a) protein-protein interactions of deoxyribonucleotide biosynthetic enzymes, and b) possible linkage between precursor metabolism and DNA polymerization. Localization of viral DNA within discrete loci in infected cells makes immunocytochemical localization a powerful technique for determining possible connections between precursor metabolism and DNA replication. With the realization that these interactions may be weak and/or transient in nature the use of anti-idiotypic antibodies was seen as a possible probe for detecting such interactions initially, with subsequent characterization of the purified proteins and their interactions to occur in vitro. At this time vaccinia virus is known to encode genes for ribonucleotide reductase, thymidine kinase, thymidylate kinase, dUTPase, DNA polymerase, and topoisomerase (Traktman, 1990) The logical enzyme for these studies for our laboratory was ribonucleotide reductase (RR) of vaccinia virus. The reasons for this were two-fold: a) RR is the central enzyme in the metabolism of deoxynucleotides for DNA metabolism since it catalyzes the first reaction committed to DNA synthesis, b) both subunits of vaccinia RR have been cloned and overexpressed in this laboratory, so that subsequent studies with purified proteins can be done.

IV.2 Materials and Methods

Generation of anti-idiotypic antisera against anti VVR2

A polyclonal antiserum was generated against R2 as described in Howell et al. (1992) The IgG fraction was obtained by passing the antiserum over a protein A-Sepharose (Sigma) column as described in Harlow and Lane (1988). Between 1 and 3 mg of anti-VV R2 IgG antibodies were injected into four New Zealand White rabbits. The rabbits were boosted once a month and bled ten to fourteen days afterwards. Two (4087 and 4093) of the four rabbits generated antibodies against a putative 34-kDa vaccinia-encoded protein. All the data presented here were generated from 4093, although most of the experiments were done with both antisera with virtually identical results.

Immunoprecipitation of radiolabeled proteins

Confluent monolayers of BSC_{-40} cells in 60-mm dishes were infected with 15 PFU/cell of vaccinia virus strain WR. At labeling periods the medium was replaced with 1 ml of MEM minus methionine with 50 µCi/ml [^{35}S]-methionine. For cells to be used for immunoprecipitating newly synthesized DNA 1 µl/ml of BUdR labeling mix (Amersham) of medium was added 20 minutes prior to harvest. After 2 hours the medium was removed and the plate washed twice with PBS. 800 µl of ice-cold RIPA (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM tris pH=8.0) lysis buffer was added and extracts were briefly sonicated to decrease viscosity. For the time course experiment 20 µl of antiserum was added to the extracts while either 5 µl of the polyclonal antiserum or 100 µl of anti-BrdUrd (Amersham) was added to the extracts, mixed and set on ice for 1 hour for the other experiments. 100 µl of a 10% slurry of protein A Sepharose (Sigma) in RIPA was added and the mixture then rotated slowly for one hour at 4° C. The beads were spun and washed three times and prepared for SDS-PAGE. After gel electrophoresis the gel was prepared for fluorography and placed under film for 2 days at -70°.

Partial Proteolysis of Immunoprecipitated Proteins

[^{35}S]methionine-labeled extract were prepared as above except that 100-mm plates labeled with 100 μCi/ml were used and multiple lanes of each immunoprecipitation were loaded on the initial SDS-PAGE. The gel was rinsed in water and dried without fixing and placed on film for two days. The positions of the bands on the gel were marked and cut out and placed in the wells of another SDS-PAGE. Various amounts of V-8 protease (Sigma) were layered on top, the samples electrophoresed three-quarters of the way into the stacking gel and allowed to digest with the electrophoresis stopped for 30 minutes. The gel was then run and processed as usual for fluorography.

Immunofluorescence with anti-anti-R2 and anti-BrdUrd

BSC_{40} cells were grown in 12-well dishes on round cover slips until nearly confluent. Cells were initially infected with between 1 and 5 plaque forming units (PFU) for two to three hours in different experiments. For cells in which newly synthesized DNA was to be localized 1μl/ml of BrdUrd labeling mix (Amersham) was added to the medium 20 minutes prior to harvesting. Cells were harvested for fixing by rinsing twice with PBS and fixing in 4% paraformaldehyde followed by permeabilization with methanol. The preimmune and immune sera were diluted 1:20 (all antibodies were diluted in PBS with 3% BSA) and incubated on the cover slips in a humidity chamber for 30-60 minutes. After three rinses (all rinses with PBS), a 1:400 dilution of biotinylated anti-rabbit antibody (Pierce) was added and incubated for 30 minutes. After three more rinses a 1:100 dilution of streptavidin-FITC was incubated on the samples. The cover slips were rinsed three times and undiluted monoclonal anti-BrdUrd (Amersham) was layered on and incubated for 1 hour, followed by three rinses. Finally a 1:100 dilution of rhodamine-conjugated anti-mouse antibody (Biomed) was incubated on the cells and rinsed three times. The cover slips were mounted in DAPCO (Sigma) on microscope slides and viewed and photographed (Ektachrome 400 film) on a Zeiss microscope with fluorescent optics and filters for fluorescein and rhodamine.

Binding of vaccinia-infected cell extract proteins to single-stranded DNA cellulose

A confluent 100-mm plate was inoculated with 20 PFU/cell in the presence of 30 μM arabinosylcytosine (araC) for one hour. The inoculum was replaced with 20 μCi [^{35}S]-methionine in MEM minus methionine with 30 μM araC and the infection was allowed to go for six hours. The cells were washed twice with PBS and spun down and resuspended in 500 mM NaCl lysis buffer and sonicated 3X for 5 seconds each time. The extract was incubated with DNase I (Sigma), dialyzed extensively against a 100 mM NaCl buffer, and centrifuged at 27,000xg for 15 minutes before being loaded on a 0.8-ml single-stranded DNA cellulose column (Sigma). Elutions of 7 ml at100 mM, 5 ml at 300 mM, 5 ml at 600 mM, 3 ml at1 M and 3 ml at 2 M NaCl were done at a flow rate of 4 ml/hr. One-ml fractions were collected and TCA precipitated with rabbit serum as a carrier protein. Bands corresponding to the ribonucleotide reductase polypeptides and the p34 polypeptide were confirmed by Western blot of labeled extracts and immunoprecipitations..

Immunoprecipitation of orthophosphate-radiolabeled BSC_{40} Cells

BSC_{40}cells were grown to 95% confluency on 60-mm plates and infected with 15 PFU/cell in medium lacking inorganic phosphate. After one hour inoculation at 37°C the inoculum was removed and 1 ml of 100 μCi $H_3{}^{32}PO_4$ containing MEM minus cold inorganic phosphate was added. The cells were harvested at 3.25 hours post-infection and treated as in the [^{35}S]-met immunoprecipitation experiments except that instead of sonicating the extracts 50 μg/ml of DNase I was added only to the extracts immunoprecipitated with the rabbit serum.

Isolating lambda gt11 recombinant expressing anti-anti R2 binding domain

A lambda gt11 library created from a partial Hae III digest of WR vaccinia virus was obtained from the laboratory of Dennis Hruby at Oregon State University. The library was screened using a 1:1000 dilution of the anti-anti R2 antibodies, and positive plaques were replated and screened again. One recombinant was cesium chloride gradient purified (Sambrook et al., 1989) and

double-stranded sequenced using the forward primer (provided by Rohrman laboratory) and Sequenase (USB).

Cloning of vaccinia ORF I-3 into pET 11c

A clone of the *Hin* d III I fragment in pBR322 was originally obtained from B. Moss. The left end 1.5-kb *Hin*d III/*Bam* HI was subcloned into pT7-7 (pT7-7/H3-BH1-1.5). A *Nde* I site was engineered in the initiator methionine for orf I-3 by generating a 100-nucleotide replacement fragment for the 5' end of the gene by use of polymerase chain reaction (PCR). An oligonucleotide 5'-GGGGGGCATATGAGTAAGGTAATCAAGAA- 3' (the underlined sequence deviates from the genomic sequence) with the *Nde* I site incorporated was used for the 5' site and the oligonucleotide 5'-GCATTAGATTACTAATCGATT-3' with a *Cla* 1 site incorporated was used for the 3' site. The PCR product and the pT7-7/H3-BH1-1.5 were cleaved with *Nde* land *Cla* I gel isolated, ligated, transformed into *E. coli* JM 83 cells. Multiple clones were picked, analyzed and were all found to have the correct insert. Numerous attempts at transforming BL21/DE3 cells with this construct were unsuccessful, indicating that even basal expression of this gene was toxic to the host cells. The I-3 ORF was dropped out of the P7-7 vector by using *Nde* I and *Rsa* I and ligated into pET11c which had been linearized with *Bam* H1, the *Bam* H1 site filled in with Klenow, and then cut with *Nde* I.

Both JM 83 and BL21/DE3 were capable of being transformed with this construct although growth of BL21/DE3 cells with the pET11c/I-3 was slowed considerably compared to pET11c alone. The recombinant gene was expressed by growing the cells to A_{595} of 0.5 at 30° C and inducing with 400 μM IPTG.

SDS-Polyacrylamide Gel Electrophoresis and Western Blots

Proteins were separated by electrophoresis on 12% polyacrylamide gels as described by Laemmli (1970). Polypeptides were transferred to immobilon-transfer membranes (Millipore, Bedford, MA) for 60 min at 400 mA with an electroblotter (Idea Scientific, Minneapolis, MN) as per manufacturer's instructions using a transfer buffer with 5% methanol. The membrane was blocked with TBST with 1% gelatin and then incubated with 1:500 dilution of

putative anti-idiotypic or preimmune antisera. A goat anti-rabbit antibody conjugated to alkaline phosphatase was used at a dilution of 1:7000 (Promega).

Purification of Recombinant p34

One-liter cultures of pET 11c/I-3 in *E. coli* BL21/DE3 were grown to a A_{595} of 0.4 and induced with 400 μM IPTG and grown for 3 hours at 30° C. The purification on single-stranded DNA agarose was a modified version of the purification of bacteriophage T4 single-stranded DNA binding protein (Bittner et al, 1979) Briefly, the bacterial cell pellet was resuspended in three volumes of high salt buffer (40 mM Tris-Cl pH 7.5, 0.5 M NaCl, 1 mM EDTA, 10 mM MgCl, 2 mM CaCl2, 1 mM B-mercaptoethanol), lysed by French press and incubated with gentle rocking for 1 hour at 20°C. The resultant lysate was dialysed extensively against 100 mM NaCl buffer (0.1 M NaCl, 20 mM Tris-Cl pH 7.5, 1 mM EDTA) and then centrifuged at 27,000 g for ten minutes. The clear supernatant was loaded on a 10- ml single-stranded agarose (BRL) column at a flow rate of 8 ml/hour. The column was washed with 0.1 M and 0.5 M NaCl buffers (20mM Tris-Cl pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol) at 30 ml/hr until the protein peak dropped to near baseline for each salt concentration. The bulk of the recombinant p34 was eluted off with a 2M NaCl buffer and was estimated to be 90-95% pure by densitometry on a Coomassie stained gel.

IV.3 Results

Anti-idiotypic antibodies to viral ribonucleotide reductase recognize a 34-kDa viral protein

Polyclonal antibodies were generated against a fusion construct of the small subunit polypeptide (R2). The IgG fraction was purifed from crude serum by using a protein A column. Two of the four rabbits injected with anti-R2 (IgG) antibodies yielded antibodies which reacted with an apparent viral protein as determined both by immunoprecipitations of labeled protein and by Western blots with crude extracts run on SDS-PAGE after infection with vaccinia virus stain WR. Both types of experiments showed that the putative anti-idiotype antibodies reacted against a virally induced 34-kDa polypeptide. Multiple experiments pulse-labeling with [^{35}S]methionine shows that the unique 34-kDa band is expressed early in infection and then dramatically decreases 5 hours post-infection (Figure IV-2). The pre-immune serum for these immunoprecipitation showed no such band, although there were two pre-immune bands at 60 and 63 kDa, which appeared late in viral infection.

Previous experiments in Chapter 3 have shown some evidence for the ribonucleotide reductase being at least transiently localized in the areas of viral DNA replication (also referred to as virosomes). In order to test this initial result further, immunocytolocalization was employed by using the anti-idiotypic antibodies which yielded the 34-kDa band and a monoclonal antibody to bromodeoxyuridine (BrdUrd) (Gratner, 1982) BSC_{40} monkey kidney cells were grown to near confluence and infected at 1 PFU per cell until viral DNA synthesis is at a maximum, varying from two to three hours post-infection. Twenty minutes prior to harvesting of the cells, labeling medium was added; this medium contains BrdUrd for labeling of newly synthesized DNA. In order to minimize any potential cross-reactivity of the secondary antibodies, the system for labeling the 34-kDa protein (anti-anti-R2) was applied first in most experiments. The results of these experiments showed a discrete localization of the anti-idiotypic antibodies to the same loci as the monoclonal antibodies to BrdUrd (Figure IV-3). This demonstrates a potential physical linkage of ribonucleotide reductase and replication of viral DNA. Experiments without the DNA labeling antibody system also showed localization to apparent viral

replication centers in the cytoplasm, while uninfected cells showed no such localization.

Establishing that 34-kDa protein is a DNA-binding protein

The results of the immunofluorescence experiments pointed to an association between RR and a viral protein localized to viral DNA replication sites. Coupled with the results from earlier experiments, the data indicate that RR itself is localized to these replication sites. Since many replication proteins are DNA-binding proteins, I performed an immunoprecipitation experiment with the anti-BrdUrd antibodies to immunoprecipitate newly synthesized DNA and any tightly associated proteins. The results showed a 34-kDa protein which comigrates with the anti-idiotypic immunoprecipitated 34-kDa protein (Figure IV-4). In cells where the BrdUrd was not added no such band can be seen, indicating that the antibody is not binding the 34-kDa protein directly.

Even though the two polypeptides are the same apparent molecular weight as determined by SDS-PAGE, there remained a distinct possibility that these immunoprecipitated proteins are not the identical protein. In order to establish this positively, the two immunoprecipitated 34-kDa bands were cut out of an SDS-PAGE gel which had been dried and autoradiographed. The dried slices were loaded on top of another SDS-PAGE gel and varying amounts of V-8 (Sigma) protease was layered on top to perform an in-gel partial proteolysis (Harlow and Lane, 1988). The patterns produced are identical (Figure IV-5), thereby establishing that the DNA binding protein is the same protein as the putative R2-binding protein.

To rule out the possibility that the protein binding to BrdUrd- substituted DNA is an artifact and test the binding of the 34-kDa protein to single-stranded DNA, a crude extract of vaccinia virus-infected BSC_{40} cells harvested for early viral proteins was run over a single-stranded DNA column which was eluted with NaCl solutions in a stepwise fashion. The putative DNA-binding protein (referred to as DNAp34 henceforth) starts to elute in significant amounts at 0.6 M NaCl with the greatest amount coming off at 1.0 M NaCl and it was the only protein still being eluted at 2 M NaCl (figure IV-6). A fraction of R2 was retained until elution with 0.3 M NaCl as determined by Western blot analysis of the fractions. Experiments with the purified recombinant vaccinia R2 subunit show

that R2 alone is not retained on a single-stranded DNA cellulose column (data not shown).

Identification of the structural gene for DNAp34

Although the DNAp34 appears after infection with virus, there still remained the possibility that the protein is a host protein that was induced by vaccinia virus infection. In order to establish if a viral gene encoded for this DNA-binding protein, a lambda phage expression library of *Hae* III restriction fragments of vaccinia virus DNA was obtained from Lisa Wilson in D. Hruby's laboratory (Department of Microbiology, OSU). Positive plaques were picked, amplified, and screened against pre-immune serum as well as immune serum. DNA sequencing of the positive lambda fusion clones revealed that the viral gene which encodes the 34-kDa protein is located in the *Hin*d III I restriction fragment of the vaccinia virus genome (Figure IV-7a and b). The open reading frame (I-3) is immediately next to the gene (I-4) encoding the large subunit polypeptide of RR. The predicted molecular weight is 30-kDa and an earlier Northern blot analysis indicated that the mRNA for this protein is present early as well as late (Schmitt and Stunnenberg, 1988). The putative promoters for the gene include both an early and an extensively studied intermediate class (Hirschmann et al., 1990).

The vaccinia virus insert in the immunoreactive lambda phage clone that was sequenced contained DNA corresponding to the last one third of the 3' end of the ORF (corresponding to the carboxy-terminal 81 amino acid residues). The epitope to which the anti-idiotypic antibody corresponds must lie within the C-terminal domain of the DNAp34 protein. From this datum it is reasonable to conclude that the interaction between ribonucleotide reductase R2 protein and the 34-kDa DNA-binding protein involves the carboxy-terminal region of DNAp34.

DNAp34 is a phosphoprotein

One of the proteins which can be isolated with the virosomes in cell fractionation experiments is a phosphoprotein which has a polypeptide molecular weight of 34-kDa. To determine if this is the same DNA binding protein infected BSC40 cells were labeled with [^{32}P]orthophosphate and

harvested at 3.25 hours post-infection. Detergent lysed cell extracts were immunoprecipitated with anti-anti-R2 serum. The results (Figure IV-8) show a 34-kDA phosphoprotein immunoprecipitates only in infected cells, demonstrating that the R2 binding protein is the same DNA binding phosphoprotein which had been localized to the virosomes previously.

Purification and initial characterization of recombinant DNAp34

The open reading frame for DNAp34 was cloned into a PET11c vector expression system and overexpressed in *E. coli* strain BL21/DE3 cells to demonstrate that this gene produces a protein of the same molecular weight as well as being detected by the anti-anti-R2 antibodies (Figure IV-9a). The recombinant DNAp34 polypeptide migrates at 34-kDa, is eluted from a single-stranded DNA cellulose column initially at 0.6 M NaCl, with the major elution peak at 2 M NaCl. The recombinant DNAp34 can be eluted from a single-stranded DNA agarose at 95% purity as an initial fractionation step in a large scale purification protocol (Figure 4-9b). The enriched recombinant DNAp34 elutes as a broad peak at 0.5 M NaCl on a native-DNA cellulose column while the majority will elute at 2 M NaCl on a single-stranded cellulose column (Figure IV-10).

When using the purified recombinant DNAp34 there is a stimulation of RR activity in *in vitro* assays of purified recombinant RR under conditions tested so far (Figure IV-11). The results of this experiment indicated a 1.5-fold stimulation of the RR activity. While the stimulation was not dramatic it was reproducible (five independent experiments) and specific (BSA control show little stimulation). Recent optimizations of the RR assay and the cloning of the vaccinia virus glutaredoxin gene may yield more dramatic results in future interaction experiments (personal communications from Slabaugh and Thresher). In addition, the phosphorylation state of the protein may play a significant role in the interactions.

IV.4 Discussion

The use of anti-idiotypic antibodies to detect protein-protein interactions for DNA precursor metabolism has yielded some encouraging results in both the vaccinia system and T4 bacteriophage. Earlier cell fractionation experiments with vaccinia virus-infected cells had given some evidence for association of the R2 subunit of RR with virosomes early in infection. Although less than 5% of the total R2 in the cytoplasm sedimented with the virosomes, one of the polypeptides that sediments with the virosome is an abundant polypeptide which has been described variously as polypeptide B (Polisky and Kates 1972, 1975; Sarov and Joklik, 1973), VDP 12 (Solonski et. al., 1978) and FP 11 (Nowakowski, et al., 1978) which is DNAp34. Immunolocalization of the RR has given some evidence for RR being localized where DNA synthesis occurs. These previous results taken together provided some evidence for RR being in the general area of viral DNA synthesis but the linkage was undefined at this point. The discovery of the DNA binding protein in this work provides the first specific linkage of ribonucleotide reductase with DNA.

There are several reasons to suspect that this is a single-stranded binding protein that is involved in DNA replication (reviewed by Chase and Williams, 1986): 1. This protein is predominantly synthesized when viral DNA synthesis is at the peak at early times in infection. 2. The affinity for single-stranded DNA cellulose is typical for other single-stranded DNA-binding proteins from *E. coli* and T4 bacteriophage. 3. The acidic carboxy terminus of DNAp34 is another characteristic of single-strand binding proteins involved in replication.

While there is very little homology between the T4 gp32 and the vaccinia DNA binding protein, there is evidence which suggests that a common physical motif may exist in at least these two systems. gp32 appears to bind a number of other viral proteins involved in DNA metabolism as well as demonstrating cooperative binding itself (Alberts and Frey, 1970). An intriguing parallel of the vaccinia DNA binding protein and gp32 is the fact that the carboxy terminus of both proteins is very acidic, shows potentially four amphipathic helices and has been implicated in recruiting other proteins to the replication fork (Krassa et al., 1991). Since the epitope to which the anti-idiotypic antibody binds to can be localized to the last third of the protein from the sequencing of the lambda fusion clones, the actual interaction site may lie in the acidic domain itself. The

immunoprecipitation experiment with [^{32}P]orthophosphate-labeled cells determined that DNAp34 is indeed a phosphoprotein, although the distribution of the phosphate groups on individual molecules of DNAp34 may vary. It is of interest to determine if the binding of RR is depended on a given phosphorylation state and how this relates to DNA replication in general. Phosphorylation and dephoshorylation could provide the mechanism for a cascade of association involved in DNA synthesis at the replication fork.

It remains to seen whether the vaccinia DNAp34 interacts directly with other proteins involved in DNA synthesis, and ribonucleotide reductase facilitates the process. A major goal to be accomplished is to reconstitute the recombinant proteins to determine what the exact role of the RR is in being able to bind to a DNA-binding protein. At this point a linkage has been establish by anti-idiotypic antibodies and a 50% stimulation of recombinant vaccinia ribonucleotide reductase activity when the recombinant DNAp34 was added. The nature of the linkage now can be pursued both by genetic means and *in vitro* biochemical methods. Experiments are currently underway to determine if the DNAp34 is directly involved in DNA synthesis (see Chapter 5). Finally, the question must be asked as to whether vaccinia virus ribonucleotide reductase channels deoxyribonucleotides into the replication fork or is there another biologically significant rationale for R2 to bind to a DNA binding protein.

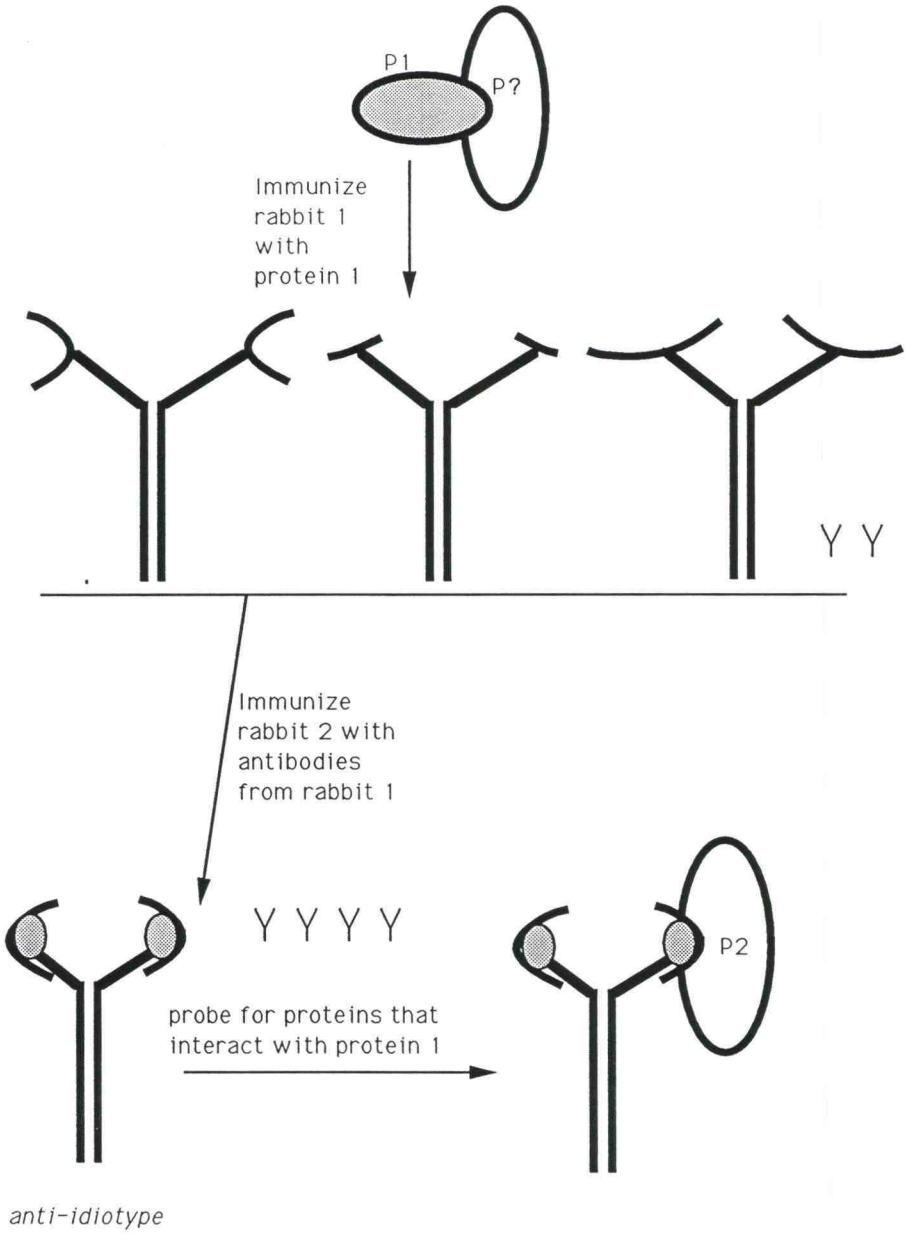


Figure IV-1 Generation of Anti-idiotypic antibodies. Anti-idiotypic can be antibodies generated to identify interactions between an enzyme and another protein (E1 and P2). A polyclonal serum contains a population of antibodies, which recognize a variety of epitopes. This population of antibodies can be used to generate anti-idiotypic antibodies which may reflect the interaction sites for protein-protein associations.

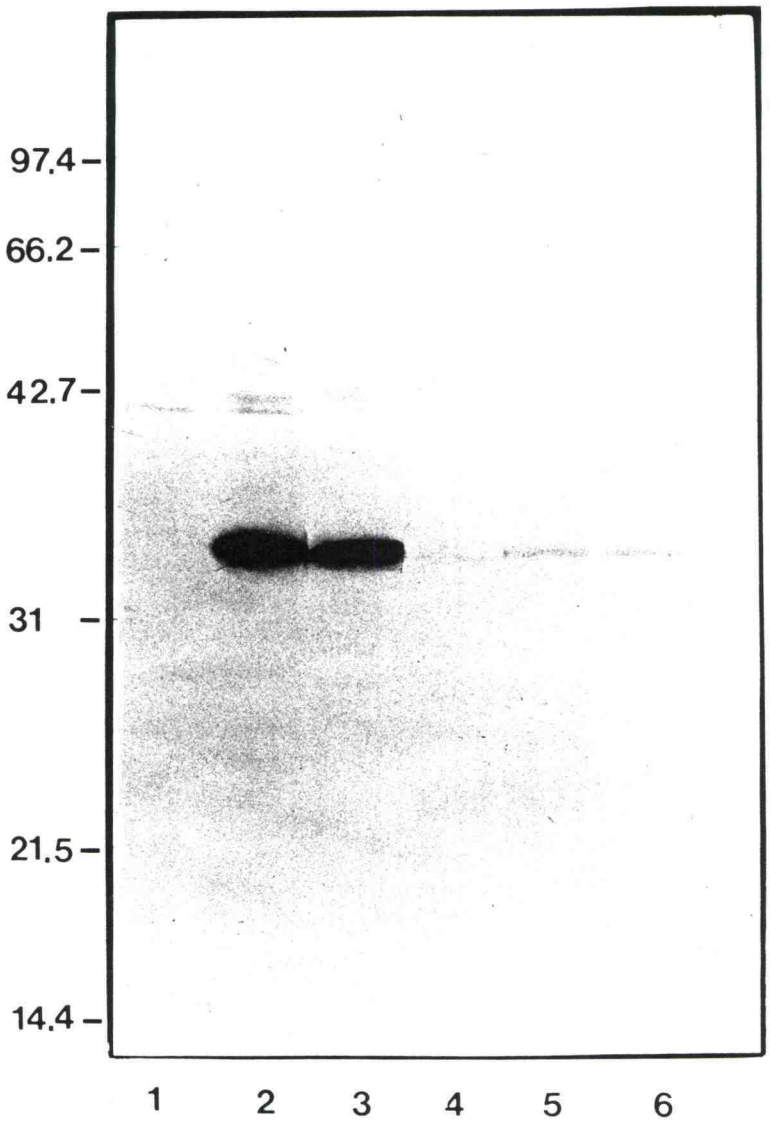
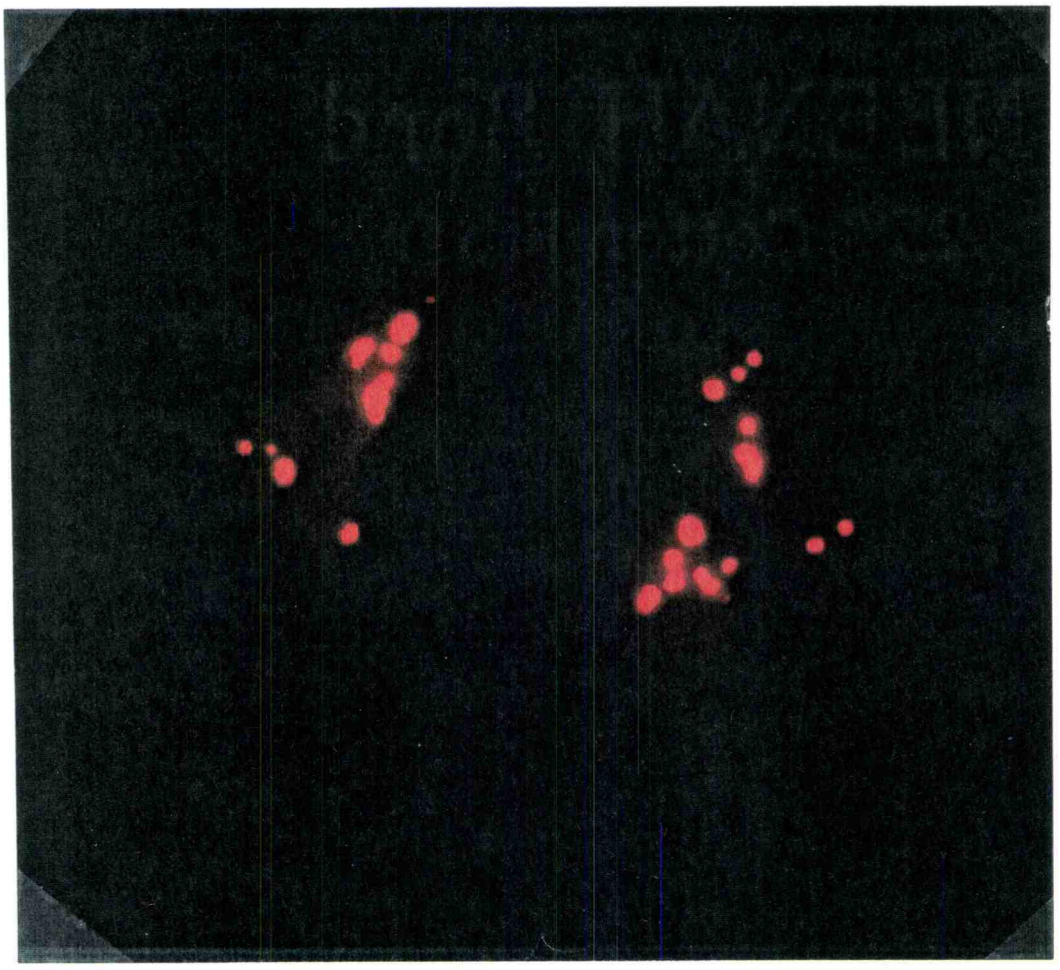
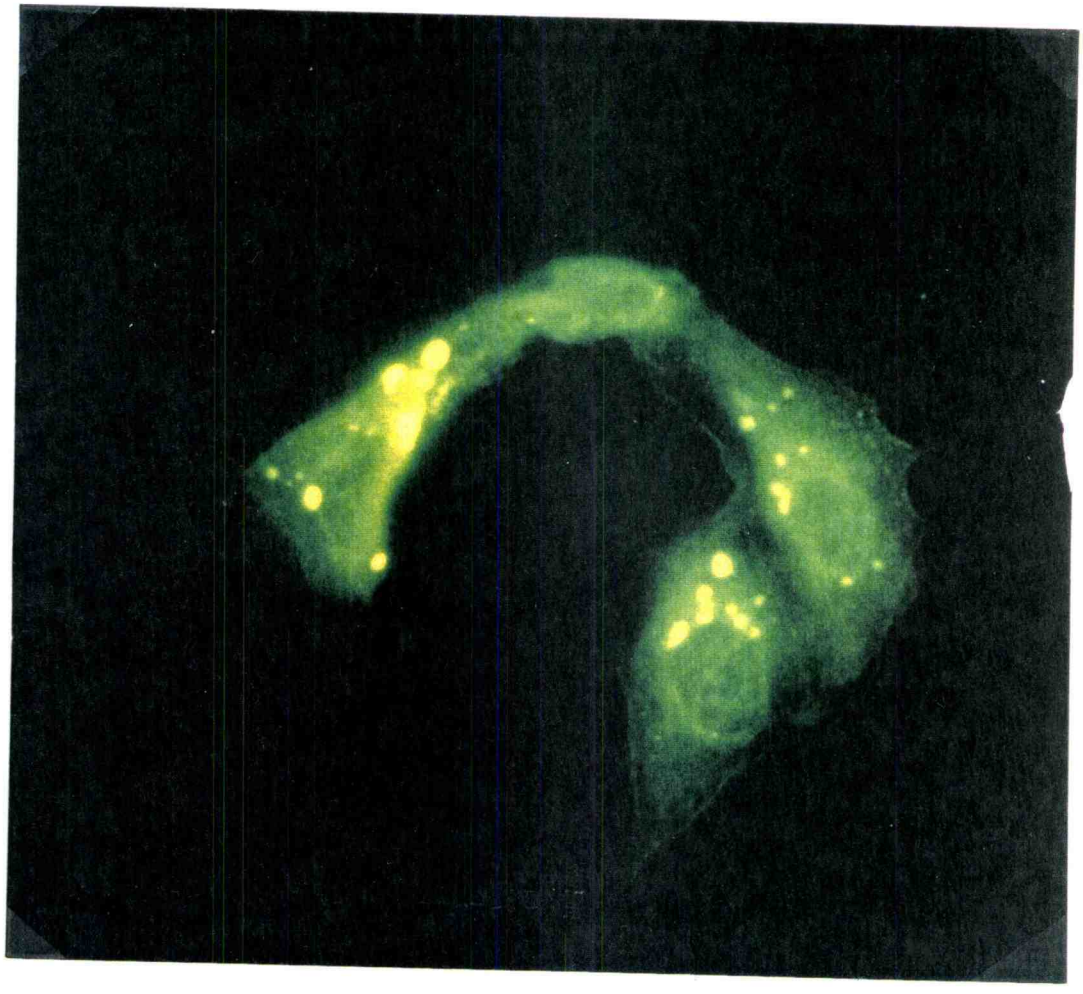


Figure IV-2 Immunoprecipitation with anti-anti-R2 of vaccinia infected (moi=20) BSC_{40} cell extracts labeled with [^{35}S]methionine for two hours prior to harvest. Lane 1 corresponds to mock-infected cells. Lanes 2-6 correspond to 2, 4, 6, 8, and 10 hours post-infection respectively.



A. Field viewed with rhodamine filter set. (Viral DNA localization)

Figure IV-3 BSC 40 infected with 1 PFU/cell and pulse-labeled with BrdUrd at 2.5 hours post-infection. Cells were fixed with methanol at 2.8 hours post-infection (p.i.). Anti-anti R2 was incubated on fixed cells followed by biotinylated anti-rabbit antibody and then fluorescein-labeled streptavidin. A mouse monoclonal antibody to BrdUrd was added and a final incubation was done with anti-mouse antibody conjugated to rhodamine.



B. Field viewed with fluorescein filter set. (DNAp34 localization)

Figure IV-3 continued.

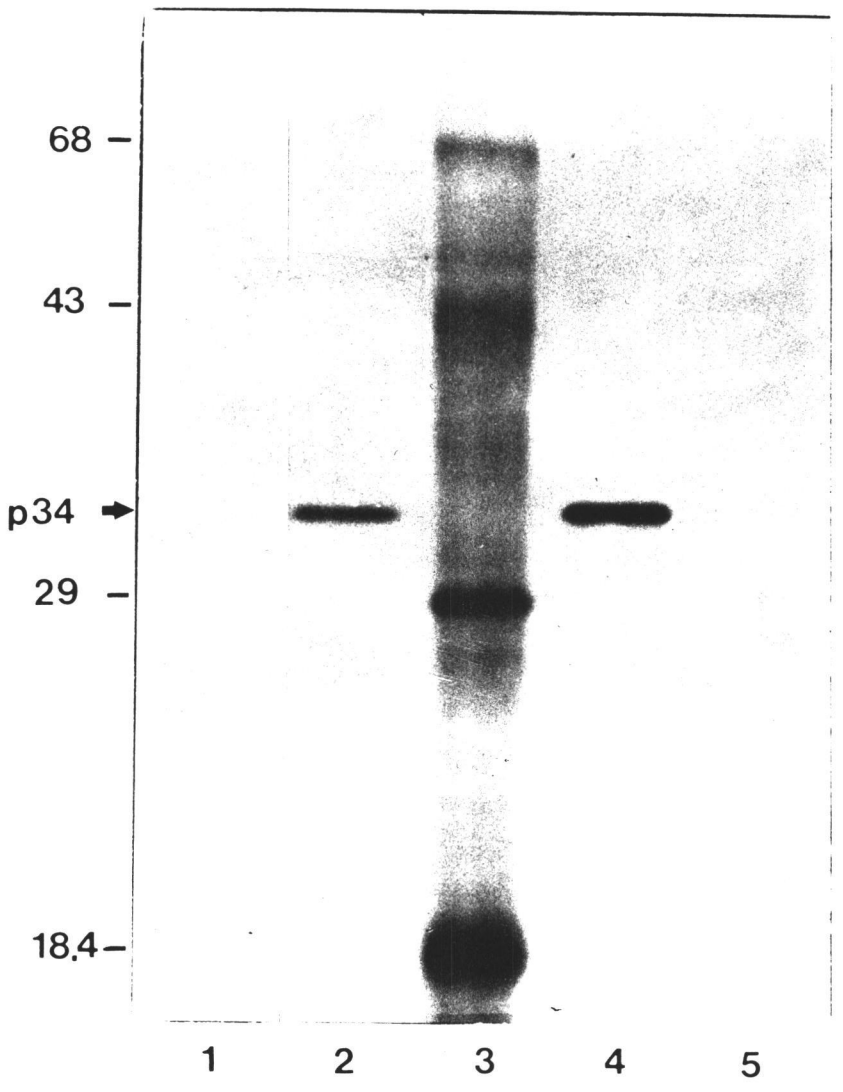


Figure IV-4 Co-immunoprecipitation of p34 protein by antibodies to BrdUrd. BSC_{40} infected with vaccinia (moi=20) and labeled from 1 to 3.5 hours p.i. with [^{35}S]methionine and labeled with BrdUrd 20 minutes (except lane 5) prior to harvest at 3.5 hours p.i.. Extracts were immunoprecipitated with pre-immune serum (lane 1) anti-anti-R2 (lane 2), or anti-BrdUrd (lane 4 and 5).

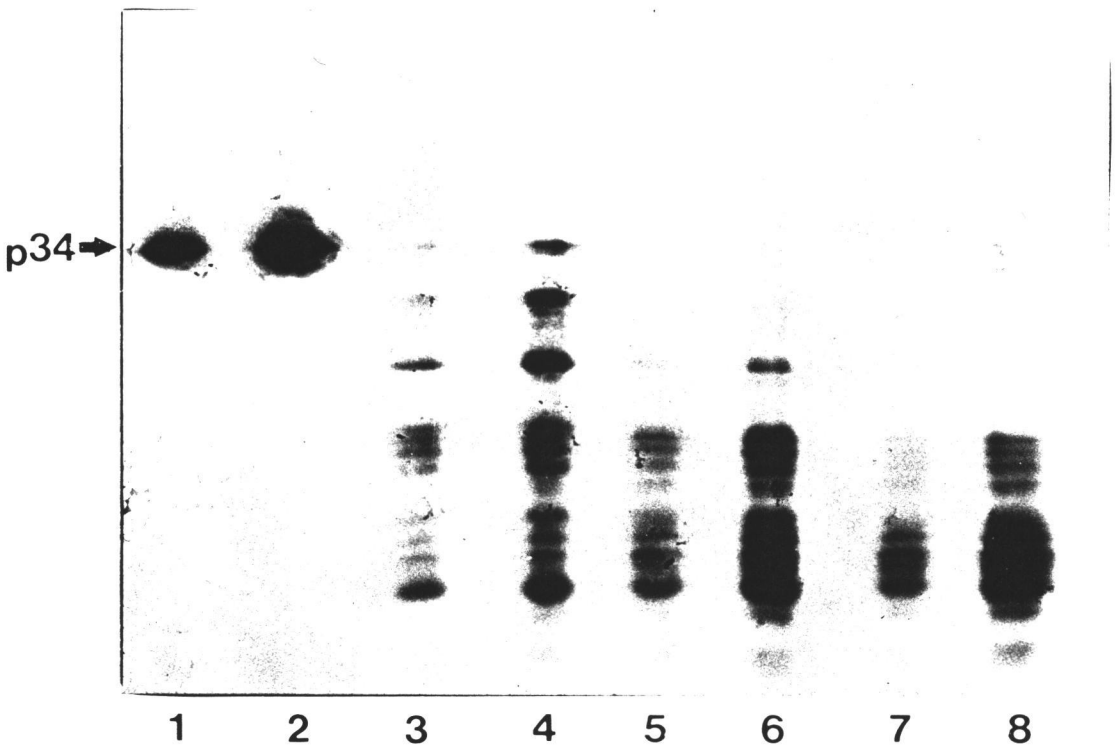


Figure IV-5 Partial proteolysis of the protein immunoprecipitated by anti-BrdUrd and anti-anti-R2. Lanes 1, 3, 5, 7 contain gel isolated 34-kDa polypeptide immunoprecipitated by anti-anti-R2. Lanes 2, 4, 6, 8 contain gel isolated 34 kDa immunoprecipited by anti-BrdUrd.

Lane 1, 2 No protease
Lane 3, 4 50 ng V-8 protease
Lane 5, 6 100ng V-8 protease
Lane 7, 8 500ng V-8 protease

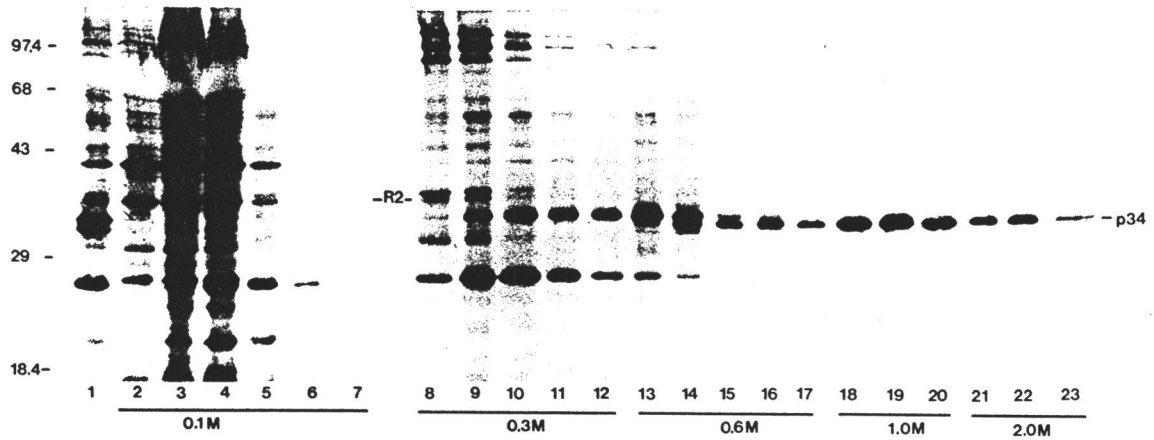


Figure IV-6 Elution of DNAp34 off of ssDNA cellulose. [^{35}S]methionine-labeled vaccinia-infected cell extracts harvested for early viral proteins were DNase'ed and extensively dialyzed and run through a ssDNA-cellulose column. NaCl steps were done at 0.1 M (lanes 2-7), 0.3 M (lanes 8-12), 0.6 M (lanes 13-17), 1.0 M (18-20), and 2.0 M (lanes 21-23) . 1 ml fractions were TCA-precipitated and run on a 12% SDS-PAGE. The DNAp34 and R2 band identifications were confirmed by Western blot and immunoprecipitations..

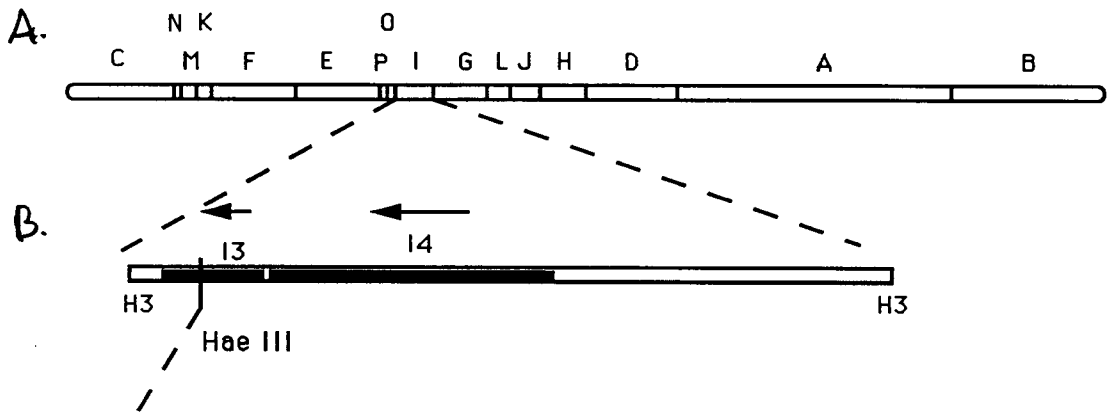


Figure IV-7 Determination of DNAp34 gene and region of protein that interacts with R2. A. Hind III restriction map of vaccinia virus WR stain genome. B. Position of open reading frames (ORF) in Hind III I fragment and fragment that was in the lambda phage expression clone. Note that I-4 is the gene for the large subunit polypeptide (R1) from RR. C. Amino acid sequence for carboxy terminus of ORF I-3. Acidic amino acids are **bolded**.

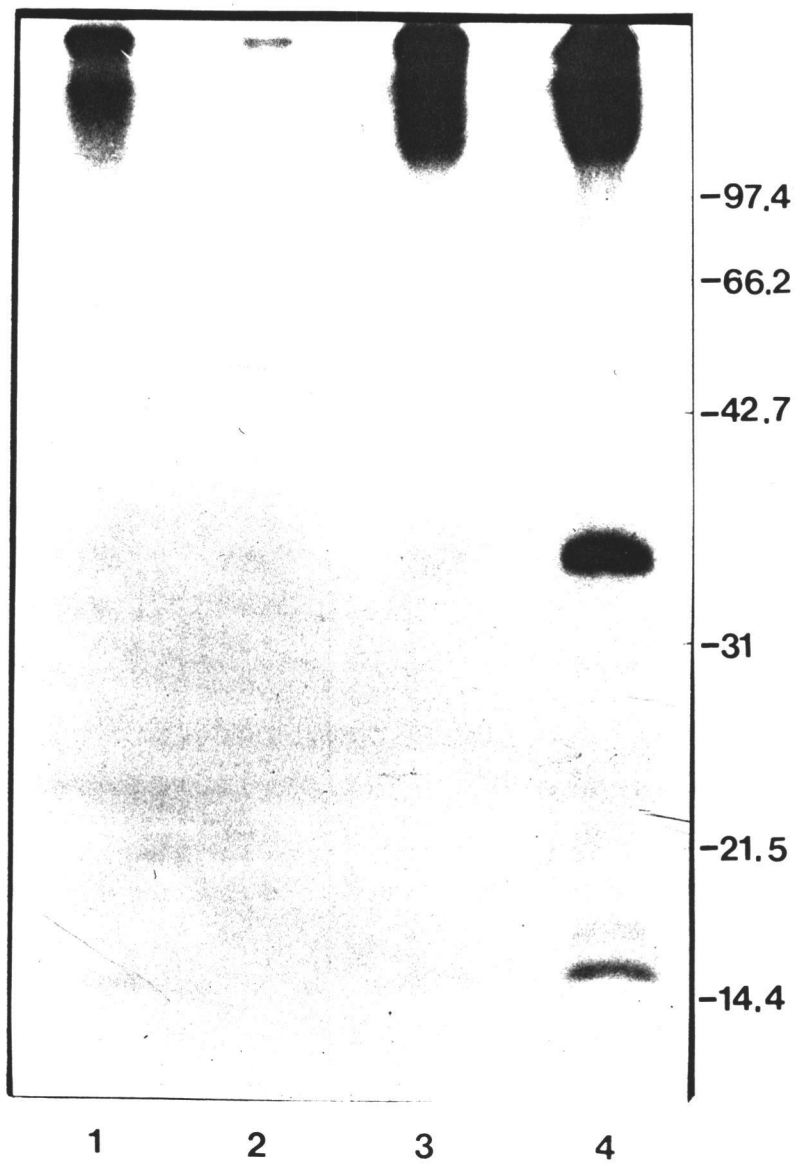


Figure IV-8 Immunoprecipitation of phosphorylated DNAp34.
BSC_{40} cells were infected with vaccinia virus (moi=15 pfu) and labeled with [^{32}P]orthophosphate from 1 to 3 hours post-infection.
Lane 1- uninfected cells extracts immunoprecipited with anti-anti-R2, lane 2 - infected cell extracts immunoprecipitated with anti-BrdUrd/ no BrdUrd added to cells, lane 3 - infected cell extracts immunoprecipitated with anti-BrdUrd/BrdUrd added 20 minutes prior to harvest, lane 4 - infected cell extracts immunoprecipitated with anti-anti-R2.

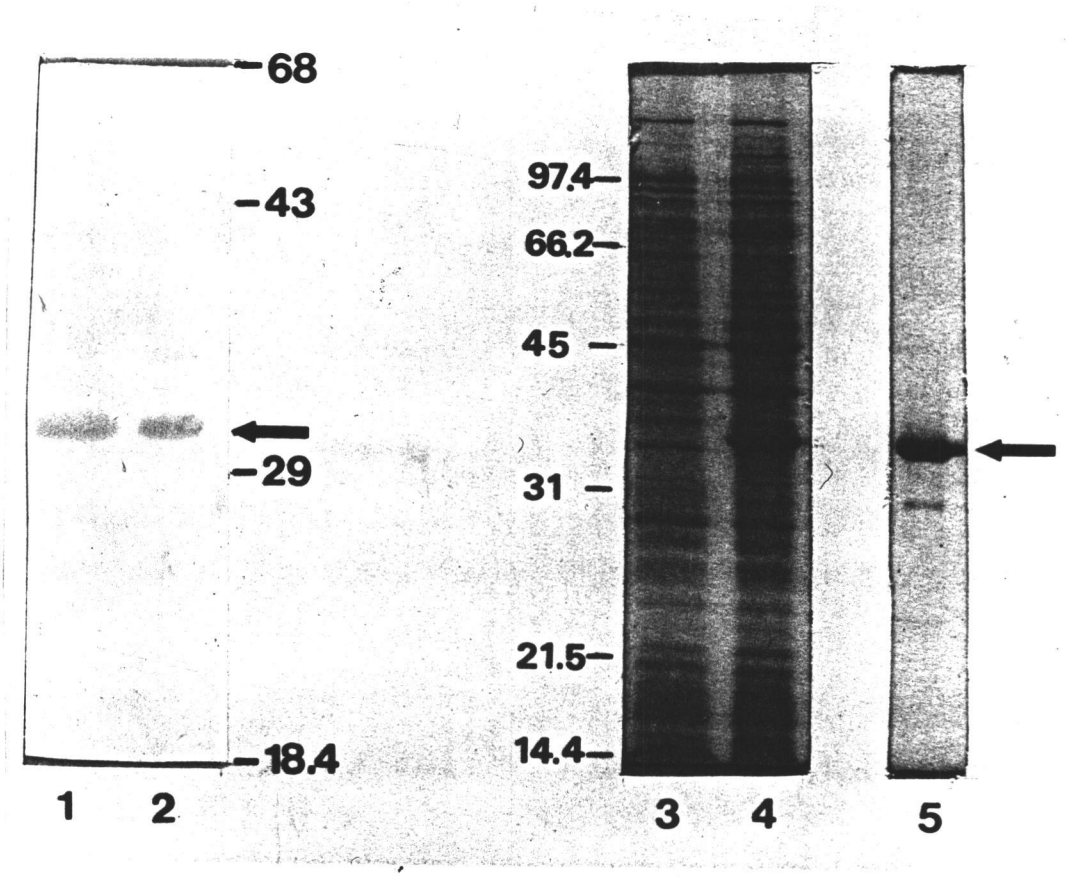


Figure IV-9 Comparision of recombinant and native DNAp34. (A) Extracts of vaccinia-infected cells and induced (IPTG) recombinant DNAp34 in a crude bacterial extract. (B) One step purification of rDNAp34 on ssDNA agarose.

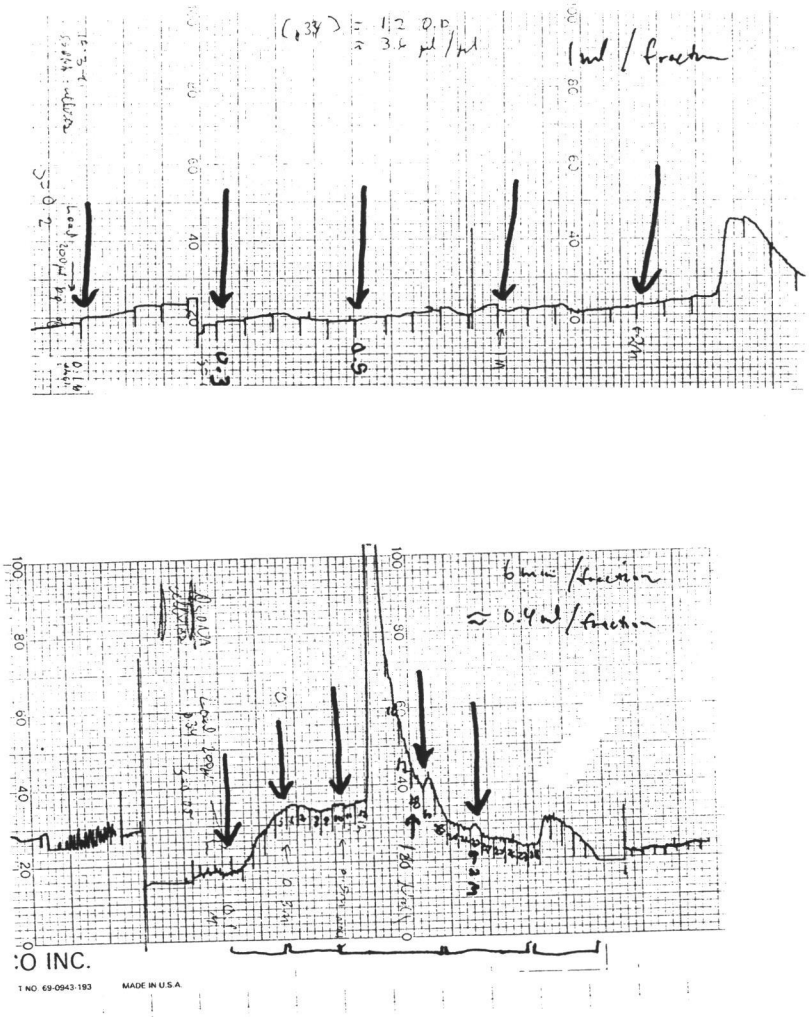


Figure IV-10 Elution of purified rDNAp34 on double-stranded and single-stranded DNA cellulose. Equal amounts of rDNAp34 were loaded onto ssDNA cellulose (A) and dsDNA cellulose (B) and eluted with 0.1 M, 0.3 M, 0.5 M 1 M, and 2 M NaCl as indicated by arrows from left to right.

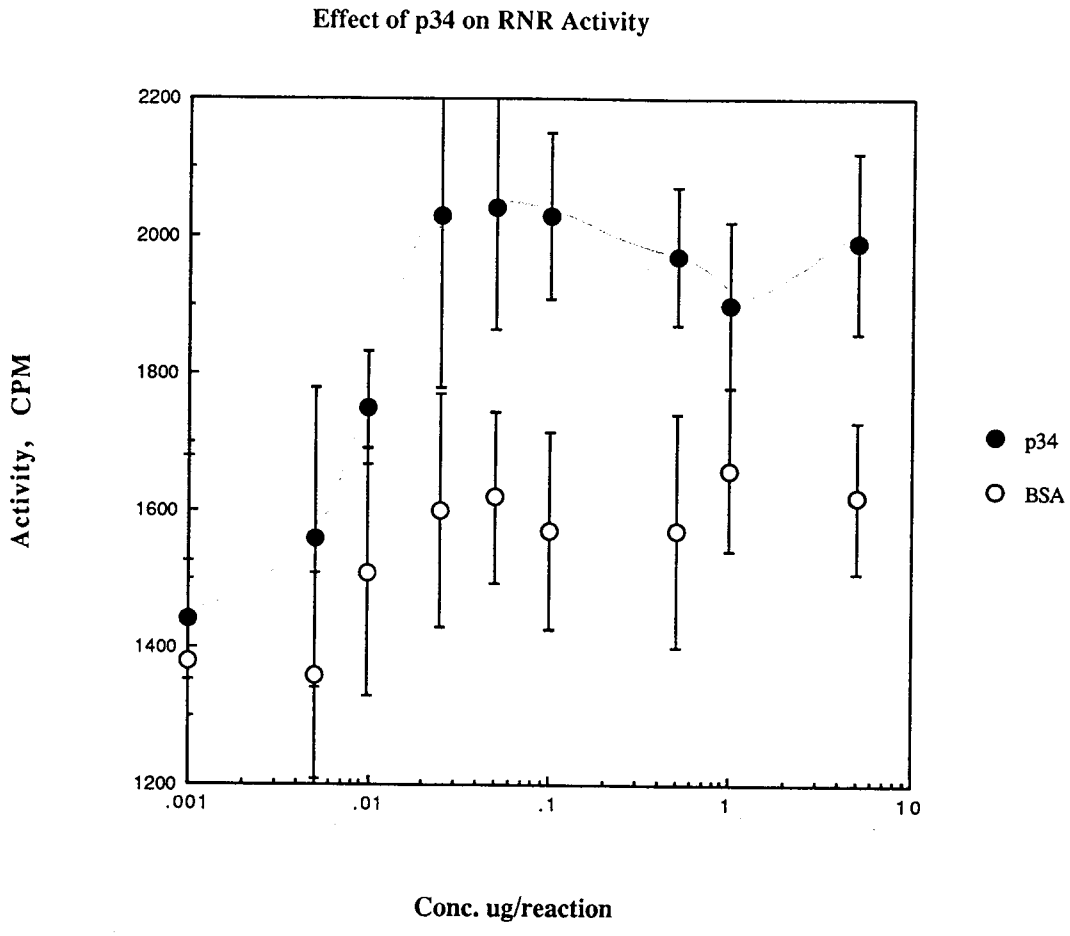


Figure IV-11 Stimulation of ribonucleotide reductase activity. Purified recombinant RR was added to recombinant DNAp34, and RR activity was assayed as in Howell et al. (1992).

Chapter V
Current Work, Future Directions and Cnclusions

V.1 Working in Progress

Inhibition of Synthesis of DNAp34 in Vaccinia Virus-Infected Cells

In order to test the putative link of vaccinia RR with the DNA replication apparatus the role of DNAp34 in the process needed to be further elucidated. Unfortunately no temperature-sensitive mutants have been mapped to the I-3 ORF. *In vitro* stimulation of the cognate DNA polymerase has been used previously to demonstrate involvement of the single-stranded DNA binding protein in DNA synthesis. These experiments are currently pursued by the laboratory of Dr. Paula Traktman at Cornell Medical School (personal communication). The Traktman group is also attempting to construct a conditional mutant (Zhang and Moss, 1991) in the I-3 gene, the ideal course of action, but a long-term project.

Anti-sense inhibition of a target gene appeared to be a potentially short term project not being pursued by others at the present time. The advantages of the anti-sense approach is the relative short amount of time to construct anti-sense oligonucleotides or anti-sense genes compared to constructing a mutant virus. If this approach is successful with an abundant early viral protein, other proteins in the vaccinia virus genome could be targeted with this technology. Once a phenotype has been established by inhibiting the synthesis of a target protein and proves to be of further interest, a conditional mutant could be constructed to assess how relative levels of the target protein to other vaccinia proteins affect the course of the infectious cycle.

There are basically two ways to use anti-sense inhibition:

1. One approach involves the generation of an oligonucleotide which is complementary to the mRNA of the target gene (for review see Cohen, 1989). One site generally targeted is the first AUG in the mRNA, the rationale being that the anti-sense oligonucleotide prevents the binding of the ribosome to the first codon in the ORF. Alternatively, others have targeted the mRNA start site to attempt to prevent the ribosome from even binding initially to the mRNA. I choose to target the initiator AUG and had a phosphorothioate oligonucleotide (S-0ligo) (5'GATTACCTTACTCATGATTAAACC3', underlined triplet is

complementary to initiator methionine codon) synthesized by the central services laboratory (CSL)(OSU Center for Gene Research and Biotechnology). The oligonucleotide was transfected into the cells byusing cationic liposome (Rose, et al., 1991) and no inhibition of the DNAp34 nor DNA synthesis was observed over a wide range of oligonucleotide concentration (3.2 nM -20uM). At this point other target sites were considered but this approach was rejected altogether due to a study by Gao, et al., 1989, which indicated a non-specific inhibition of viral DNA synthesis in HSV by S-oligos .

2. The other approach uses anti-sense RNA generated off of a plasmid. The plasmid is transfected into a cell and the anti-sense RNA is synthesized by a RNA polymerase. The vaccinia system generated by the Moss group (Fuerst, et al., 1986; Elroy-Stein and Moss, 1990) allows the anti-sense RNA to be synthesized off of a T7 RNA polymerase promotor. The T7 RNA polymerase is provided either by a recombinant vaccinia virus (vTF7-3), or the cells (OST7-1), or both. Oligonucleotides with restriction sites compatible with the polylinker in pTZ18U (USB) were designed to flank the region of the gene which is expressed as anti-sense RNA. The target DNA was amplified by PCR, restricted and cloned into a plasmid with the T7 promoter (see Figure V-1 for details).

The results to date showed about 50% attenuation of the DNAp34 polypeptide when pulse-labeled with [^{35}S]methionine at 2-3 hours post-infection. DNA synthesis was unaffected in these experiments. Preliminary results using both VVT7 and OST7-1 cells show a decrease of viral DNA synthesis by both immunofluescence and radiolabeling newly synthesized DNA (Figure V-2). The relative level of the DNAp34 is currently being investigated.

Further Purification of Recombinant DNAp34

Analytical ultracentrifugation of DNAp34 purified on the single-stranded DNA agarose column revealed the presence of low molecular weight contaminants even after extensive dialysis with a 25,000 dalton cut-off membrane. The ratio of A_{280}/A_{260} revealed a substantial amount of nucleic acid present. Further purification is necessary to allow the physical characterization of DNAp34. Preliminary results indicate the use of a reverse phase column (phenyl sepharose on the FPLC) will produce DNAp34 that is devoid of nucleic acids and 99% homogeneous.

Biophysical Experiments to be Completed

The next preparation of DNAp34 will be used to carry out two biophysical experiments: 1. The determination of the native molecular weight of the protein both by molecular sieve chromatography and analytical ultracentrifugation. 2. The characterization of the secondary structure by circular dichroism spectroscopy.

V.2 Future Work and Conclusions

The results in this thesis suggest that indeed there is a further level of integration of deoxynucleotide metabolism at least in the vaccinia virus replication cycle. While no piece of data is beyond question the results *in toto* indicate that there are weak interactions which give rise to another level of organization. Modulation of protein-protein and protein-DNA interaction may occur as a result of the phosphorylation of DNAp34. The enhancement by ATP and dATP of RR cosedimentation with the virosome (seen in Chapter 3) may be a result of increased phosphorylation of the DNAp34 *in vitro.* Other examples of phosphorylation modulating interactions include DNA polymerase α (Nasheuer et al., 1991). Phosphorylation decreases affinity for DNA of a high mobility group I nonhistone chromatin protein (Nissen, et al., 1991) It is also intriguing that one of the three temperature-sensitive mutants which are DNA minus in the vaccinia virus genome has been determined to encode a protein kinase (Traktman, 1990).

The exact function and nature of the interactions remain to be explored further. The further characterization of RR and DNAp34 will allow questions of what conditions enhance or disrupt interaction to be addressed: What does phosphorylation of the DNAp34 do to its interaction with RR? If DNAp34 does bind to DNA cooperatively will RR bind to all molecules of DNAp34 on the DNA, or will it bind only to the end where the replication apparatus is located? Does channelling of deoxynucleotides occur? Are other proteins involved (e.g. glutaredoxin)? I would like to propose a testable model involving these questions: The DNAp34 and DNA could be considered as at least part of a scaffold around which the replication apparatus assembles. The acidic

terminus of the DNAp34 acts to recruit other proteins to the area as is thought to occur with bacteriophage T4 single-stranded DNA binding protein and some eucaryotic transcription factors. The association of individual proteins with DNAp34 may be transient and act as a nucleation site for assembly of the replication complex. Disruption of potential protein interactions with the DNAp34 could be achieved using *in vivo* competition with an overexpressed peptide homologous to the acidic terminus. This experiment was successfully performed with the carboxy terminus of gp 32. The results demonstrated that the peptide could indeed titrate away protein from the DNA replication apparatus and squelch DNA synthesis (L. Gold, personal communication). The availability of a vaccinia host cell line expressing T7 RNA polymerase would allow the preloading of host cells with the competing polypeptide prior to infection. Co-expression of an exogenous surface antigen driven off of a vaccinia promotor would allow individual cells to be sorted out which received high quantities of the transfected plasmid and were infected.

Further fine mapping of the anti-anti-R2 binding site would allow the determination of the exact site on DNAp34 where R2 interacts. A series of peptides could be synthesized to specifically inhibit the interactions of the two proteins while, conceivably, leaving other protein interaction sites intact. *In vitro* experiments utilizing analytical affinity chromatography would be useful in determining the K_d for the peptides as well as the native protein in different phosphorylation states. With the optimized peptide *in vivo* experiments could be performed to determine the biological consequences of delocalization of RR from the virosome. While the outcome in tissue culture experiments would be predicted to be subtle due to the non-essential status of vaccinia RR, it is not known if the host RR is also recruited to the virosome. There is controversial evidence that vaccinia recruits nuclear proteins to the viral replication centers. In addition, most evidence to date indicates that the host RR is localized either in the cytoplasm (Engström et al., 1988; Kucera and Paulus, 1986) or perhaps perinuclearly (Sikorska, et al., 1990). Given the high homology (80% identity) between mouse and vaccinia R2 it would not be surprising if the host RR is also recruited to the replication fork of viral DNA. If a specific peptide could be developed to inhibit interactions, processivity of the replication apparatus and mutagenesis could be assayed for relatively easily.

Finally, the work in this project points toward new avenues to explore the DNA replication apparatus using both affinity chromatography and anti-idiotypic

antibodies. Generation of antibodies to both the whole DNAp34 and the carboxy terminus should yield high titer antibodies for the previously mentioned *in vivo* competition experiments as well as, providing the antigens for the generation of anti-anti-DNAp34. Other replication proteins interacting with DNAp34 will be identified. The generation of a series of affinity columns with immobilized DNAp34 differing in phosphorylation states is an intriging variation on the approach taken by others. Lastly, the results showing that a DNA-binding protein could be co-immunoprecipitated with newly synthesized DNA suggests, the procedure could be modified to pull out other proteins in the DNA replication apparatus. By immobilizing anti-BrdUrd antibodies to a column, newly synthesized DNA and associated proteins would be preferentially retained and enriched. While most of the immunoprecipitation results with this system yielded one predominant radiolabeled protein, the fact that a buffer with detergents and moderate ionic strength was used leaves room for modications which would yield more parts of the DNA synthesis machine.

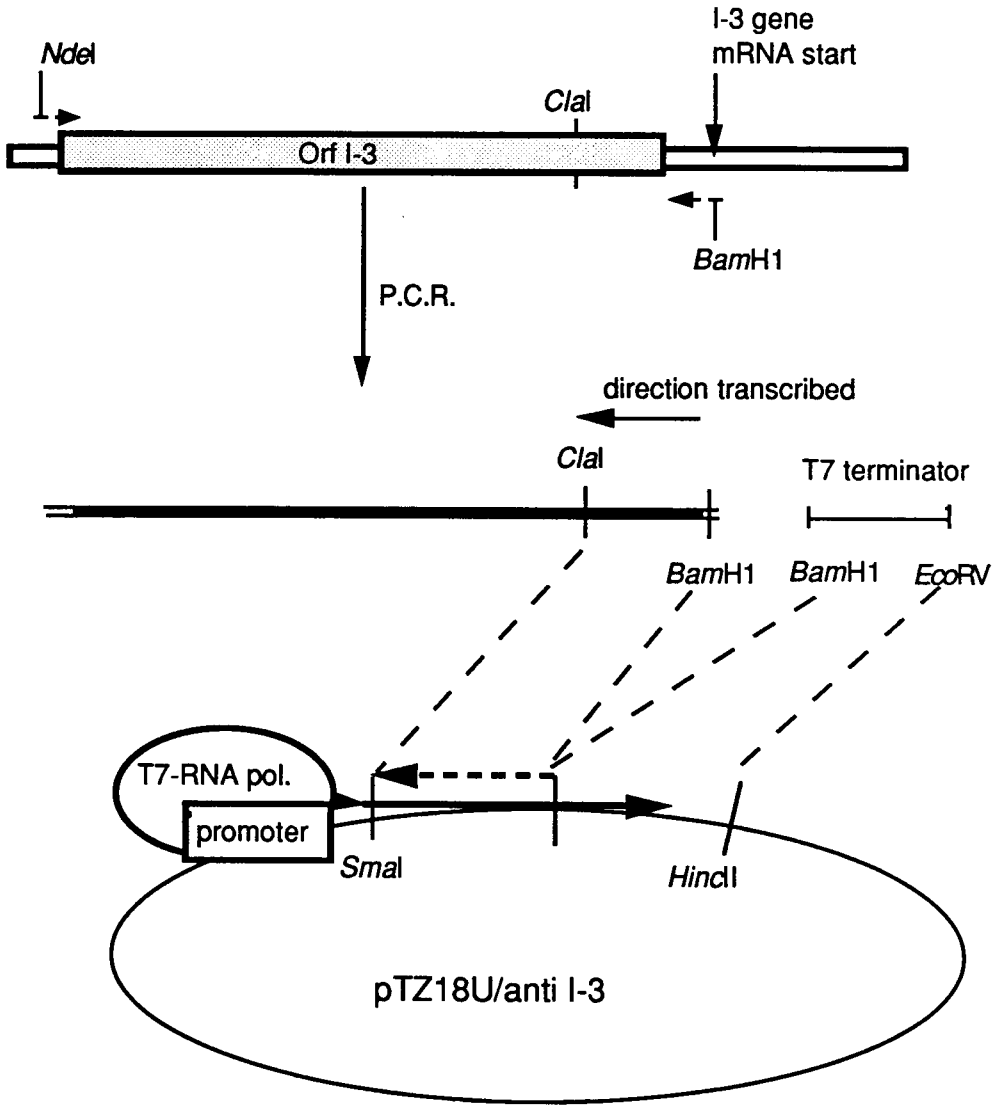


Figure V-1 Generation of anti-sense gene designed to inhibit synthesis of DNAp34. A plasmid was construct with the 5' end of the I-3 gene transcribed off of the T7 polymerase promotor in the vector when transfected into an appropriate host (OST 7-1) infected with VV T7-3..

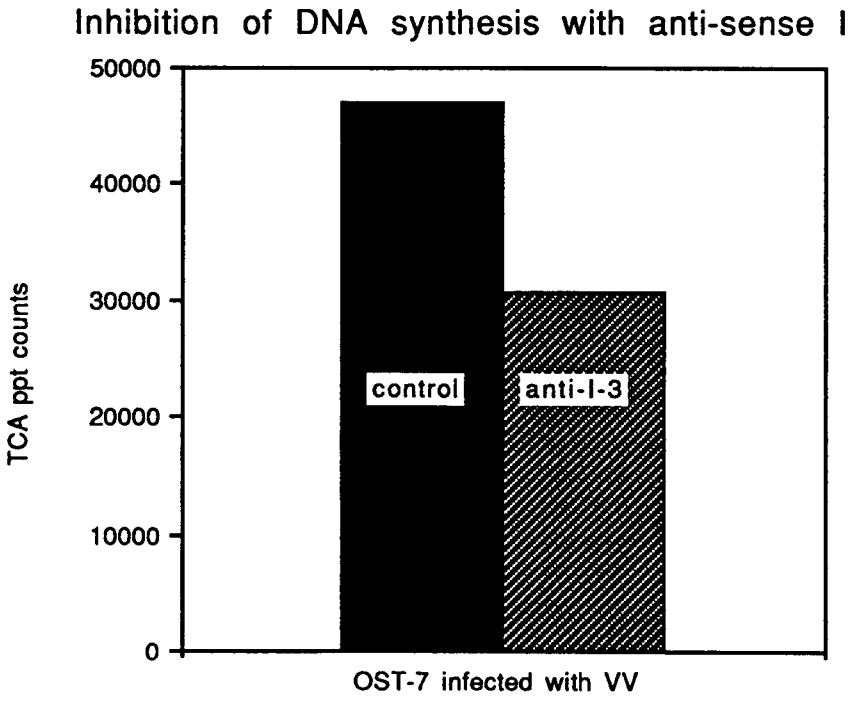


Figure V-2 Levels of DNA synthesis in cells transfected with control and anti-sense DNAp34 plasmids. OST 7-1 cells were transfected 5 hours prior to infection with 20 µg/ml of plasmid DNA with 20 µl/ml of liposomes. After infecting with 30 PFU/cell for 4 hours the cells were pulse-labeled for 15 minutes with [3H]thymidine and then harvested by TCA precipitation.

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