To the Graduate Council:

I am submitting herewith a thesis written by Thomas J. Savin entitled "Studies on Nonhistone Chromosomal Proteins." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biomedical Sciences.

W. K. Yang, Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

STUDIES ON NONHISTONE CHROMOSOMAL PROTEINS

A Thesis

Presented for the

Master of Science

Degree

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Thomas J. Savin

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ABSTRACT

In the first part of this study, nonhistone chromosomal proteins were examined for possible age-associated alteration in Fisher 344 rats. Various nonhistone chromosomal protein fractions were isolated from brain and liver tissues of 4- and 29-month-old rats and analyzed either by two-dimensional gel electrophoresis or by sodium dodecyl sulfate-polyacrylimide gel electrophoresis. I detected no age-related differences in the nonhistone protein complement. However, tissue-specific differences (between the liver and the brain) and sex-specific differences (between male and female rat brains) in nonhistone proteins were revealed. The 5 M urea soluble nonhistone protein fraction isolated from male rat brain contains 12 detectable proteins that are not present in the similar fraction from the female.

In the second part of this study, possible alteration of nonhistone chromosomal proteins during infection of NIH 3T3 cells with murine leukemia virus (MuLV) was investigated. Sucrose gradient analyses of viral DNAs reveal that MuLV DNA is in the form of a nucleoprotein complex, which was distinctly different from the minichromosome form found with the SV40 viral DNA. It was also found that circular forms of MuLV DNAs could be extracted from host chromatin with 0.35 M NaCl. This is the same salt concentration that removes a large portion of nonhistone proteins from chromatin. This suggests the possibility of interaction between viral DNA and loosely bound nonhistone proteins. Also, various cellular protein fractions, including the nonhistone protein fractions,

were isolated from NIH 3T3 cells which were uninfected, acutely infected, and chronically infected with MuLV. These proteins, which were ^{3}[H] leucine labeled, were subjected to various electrophoretic analyses. Numerous differences in the protein complement isolated from the three groups of NIH 3T3 cells were observed. The major differences were present between the chronically infected cell group and the other two groups of cells.

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LIST OF ABBREVIATIONS

Nonhistone Chromosomal Proteins	NHCP
High Mobility Group	HMG
Sodium Dodecyl Sulfate	SDS
Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis	SDS-PAGE
Phenylmethylsulfonyl Fluoride	PMSF
Ethylenediaminetetracetate	EDTA
Murine Leukemia Virus	MuLV
Eagle's Minimal Essential Medium	EMEM
Diazobenzyloxymethyl	DBM

INTRODUCTION

In the nucleus of all eukaryotic cells there exist two major groups of proteins associated with DNA: histone and nonhistone proteins. Histones are highly conserved proteins whose primary function is packaging the cell's DNA. The nonhistone chromosomal proteins (NHCP), on the other hand, include a number of functional proteins, such as DNA and RNA polymerases and their associated protein factors; DNA repair enzymes (endonucleases, exonucleases, and ligases); methylases; ribonucleases; acetylases; de-acetylases; phosphatases; phospho-kinases; nuclear pore proteins; heterogenous RNA transport proteins; ribosomal RNA transport proteins; scaffold proteins; regulatory proteins; ubigutine; and the HMG (high mobility group) proteins. Determination by two-dimensional gel electrophoresis has revealed at least 450-500 varieties of nonhistone proteins in a nucleus (Peterson and McConkey, 1976, and Macgillivray and Rickwood, 1974). Most of these proteins were found to be present in 500 to 2000 copies per haploid genome (Peterson and McConkey, 1976).

A major problem in early studies of nonhistone proteins appeared to be the complete separation of these proteins from DNA and histone proteins. Several chromatin fractionation schemes have been devised by different laboratories. Some of the fractionation methods relied on acid extractions, which solubilize the histones from the DNA. The "acidic proteins" could then be separated from DNA by phenol (Teng et al., 1971), detergent (Elgin and Bonner, 1970), or salt (Benjamin and Gellhorn, 1968). Other fractionation methods included 1 M NaCl salt extraction (Wang,

1967); extraction with a variety of salt solutions, both in the presence and absence of urea (Hill et al., 1971, and Levy et al., 1972); and dissociation by sodium dodecyl sulfate (SDS) (Shirey and Huang, 1969). Most of these methods achieved extraction of selective protein species only, but could not recover the whole population of nonhistone proteins, nor was the selective extraction the same with various tissues. Recently, the basic procedures mentioned above have been modified and improved. Specific methods are also available for the isolation of particular proteins. For example, DNA-cellulose chromatography is used to remove DNA binding proteins (van den Broek, 1973). One of the most used procedures for fractionating chromatin to extract nonhistone proteins is that of Chiu et al. (1978), which combines a variety of salt solutions, both in the presence and absence of urea, various phosphate buffers, and hydroxylapatite column chromatography. By this procedure, chromatin proteins are fractionated into three groups: the nonhistone proteins which are loosely bound to chromatin (0.35 M NaCl-5 M urea soluble); the histone proteins [2.5 M NaCl-5 M urea-1 mM sodium succinate (pH 5.0) soluble]; and the tightly bound nonhistone proteins [2.5 M NaCl-5 M urea-50 mM Tris-HCl (pH 8.0) soluble]. Nonhistone proteins consisted approximately of 90-95% as the loosely bound fraction and 2-4% as the tightly bound fraction.

The vast interest in studying the nonhistone group of proteins has been based on the consideration that these proteins could be regulators of gene expression in eukaryotic cells. One study that focused attention on this possibility was by Paul and Gilmour (1968). They demonstrated

that chromatin reconstituted from a mixture of DNA, histone, and nonhistone chromosomal proteins served as a more active template for RNA polymerase than that reconstituted from DNA and histones alone. Only the addition of nonhistone chromosomal proteins could result in the synthesis of RNA molecules with sequences that showed homology with normal RNA found in the tissue of origin of the nonhistone proteins. Since these experiments were performed, researchers have focused their attention on particular groups of nonhistone proteins, trying to elucidate their properties and how they may relate to transcription control. One such group under study is the phosphoproteins. They represent a significant proportion of the chromosomal nonhistone proteins. It is thought that some functions of nonhistone proteins may be regulated by whether or not they are phosphorylated, similar to the way that the functions of numerous cytoplasmic proteins are regulated. It has been found that some phosphoproteins are heterogeneous and tissue-specific (Platz et al., 1970, and Rickwood et al., 1973), that they can alter the rate of RNA synthesis in vitro (Spelsberg and Hnilica, 1969, and Kostraba and Wang, 1972) and that they bind specifically to homologous DNA (Teng et al., 1971). Also, in a variety of systems, changes in the phosphorylation of chromosomal nonhistone proteins appeared to be correlated with activation of gene activity (Kleinsmith, 1974). For example, studies by Chiu et al. (1975a) demonstrated that neoplastic transformation was accompanied by intensive phosphorylation of several nonhistone proteins in rat hepatoma cells. These findings of function-related variations in the phosphorylation of these nonhistone proteins are consistent with the

proposed role as gene regulators. Direct evidence for a cause and effect relationship between nuclear phosphoproteins and the activity of specific genes remains to be proven, such as direct demonstration that the presence or absence of one specific phosphorylated protein or dephosphorylated protein can alter the activity of a specific gene.

Phosphorylated nonhistone proteins are by no means the only variety implicated in gene regulation. The tightly bound proteins as well as some of the loosely bound proteins are not significantly phosphorylated but do seem to have an effect on transcription (Chiu et al., 1975b). These proteins have been shown to be tissue-specific (Chiu et al., 1975b; Wang et al., 1976; and Miyazaki et al., 1978) and species-specific (Wu et al., 1975). Using the chicken erythrocyte system, Gates and Bekhor (1979a, 1979b) performed several experiments which suggest that the tightly bound nonhistone chromosomal proteins are exclusively associated with unique sequence DNA, and that the unique sequence DNA is an incomplete subset of total chicken unique sequence DNA. These investigators were able to enrich the content of globin DNA by isolating the DNA associated with the tightly bound proteins of chicken erythrocytes, whereas the globin gene sequences were not enriched by the same isolation procedure using the tightly bound protein of chicken liver. This suggests that actively transcribed regions of DNA in a tissue have specific regulatory proteins bound to them.

The tightly bound proteins mentioned above and some loosely bound proteins have also been implicated in a role as steroid acceptors (Nyberg et al., 1976). It is known that the transfer of androgens into the

nucleus involves an androgen receptor-complex, and that this complex, which is steroid and target tissue-specific, interacts with a chromatin acceptor which is 0.35 M NaCl soluble, as well as a 2.0 M NaCl insoluble protein (Wang, 1978). One way of explaining the presence of more than one acceptor in a target organ chromatin is to consider the multiple acceptor theory suggested by Gorski and Gannon (1976). The 0.35 M NaCl soluble acceptor may be envisioned to act on DNA, activating initiation of RNA synthesis. The tightly bound proteins, which are bound to unique sequences, would function as the second acceptors. Such a binding may cause a destabilization of DNA-nonhistone interactions. The combined effect of the loosely and tightly bound acceptors would result in an altered state in chromatin, thereby facilitating an increase in RNA synthesis.

It was reported that some loosely bound nonhistone proteins seem to interact with DNA to cause alterations in its structure. The prime example of this type of interaction is that of the HMG proteins 14 and 17. These proteins are able to confer upon actively transcribed DNA a certain conformation which allows the DNA to be preferentially digested by DNase I. If these proteins are eluted with 0.35 M NaCl, the actively transcribed DNA region is not preferentially digested (Weisbrod and Weintraub, 1979).

From these examples reported above it can be seen how diverse and complicated is the role nonhistone proteins may play in the regulation of gene transcription. Although most of the evidence is circumstantial, as mentioned earlier concerning the phosphorylated nonhistones, it at least

shows the potential of this field of research. This thesis is concerned with the possible alterations of nonhistone proteins in two kinds of abnormal conditions of the cell, namely, in the aging process and during acute infection with retroviruses.

PART I

STUDIES ON NONHISTONE CHROMOSOMAL PROTEINS AND THEIR POSSIBLE RELATION TO AGING

CHAPTER I

INTRODUCTION

A number of theories of aging have been proposed. The majority of these postulate that the breakdown or change of certain cellular components, mainly DNA or protein, which are essential for survival of the cell, occurs during the aging process. The somatic mutation theory (Curtis, 1966) emphasizes the possible changes in a cell's DNA such as strand-breaks and cross-linking as the animal ages. When an essential sequence of DNA is altered to the state that it can no longer be transcribed, the cell dies. The error catastrophe theory (Orgel, 1963) predicted that random mistakes in genetic translation, initially a low frequency event, could result in a gradual accumulation of protein errors with the advancing of age. Errors in various enzymes of the protein-synthesizing apparatus would be self-propagating and could eventually lead to an error catastrophe that would be lethal to the cell. The free radical theory (Harmon, 1960) predicts that free radicals can alter or destroy biological activities of DNA and protein, which may not be repaired and hence accumulate as the organism ages. Some examples of free radicals are the hydroxyl-free radical, which has the property of causing 5-6 double bond formation in thymidine (Lagercrantz, 1973) and destroying the biological activity of the DNA, and superoxide, which is active in deamination (Anbar and Pecht, 1969). There are also the posttranslational theories of aging. These theories state that protein modification may alter its function so that it no longer performs its

assigned task. For example, methylation of an enzyme may stop it from functioning. These inactive enzymes may accumulate as an organism ages.

Of particular interest to this thesis work is the possibility that an alteration in nonhistone chromosomal proteins and their functional properties during the aging process. As mentioned previously, nonhistone proteins have been implicated in gene regulation. The theory states that if there is a physical change in a nonhistone (gene regulatory) protein, it may not be able to regulate a specific gene; therefore, the gene sequence may not be able to be transcribed, or it may be transcribed nonspecifically. A recent study suggests that the latter may be true. Ono and Cutler (1978) have found that as a mouse ages, genes which are not normally transcribed become expressed. They found an increase in the content of globin mRNA in the brain and the liver of old mice. The globin gene is not normally expressed in these tissues of a young animal. In an effort to find age-associated changes in nonhistone chromosomal proteins, Medvedev et al. (1979) compared the 0.35 M NaCl soluble nonhistone proteins of young and old rat liver chromatin and found that liver cells of old rats contained an increased number and quantity of high molecular weight proteins. It should be noted that he compared these proteins by using the method of SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

In the present study, I have compared the complete complement of nonhistone proteins isolated from old and young Fisher 344 rat brain and liver tissues by employing two-dimensional gel electrophoresis, which is considered to be superior to the SDS-PAGE because it separates proteins on the basis of charge differences.

CHAPTER II

MATERIALS AND METHODS

Preparation of nuclei from Fisher 344 rat brains and livers. All procedures involved in the isolation of NHCP were performed at 2-4°C. The procedure used was that of Hnilica and his co-workers (Chiu et al., 1978) with some modifications. Rat brains or livers (15 gm) were homogenized in a 150 ml solution of 0.32 M sucrose/5 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride (PMSF) in a Teflon-glass homogenizer using 10-12 strokes at 1000 rpm. After filtration through four layers of gauze, the homogenate was centrifuged at 1000 g for 10 minutes. The nuclear pellet was then suspended in 150 ml of 2.2 M sucrose/5 mM $MgCl_2$/ 1 M PMSF, and centrifuged at 75,000 g for 1 hour. The purified nuclear pellet was suspended in a solution of 0.32 M sucrose/5 mM $MgCl_2$/1 mM PMSF and collected by centrifugation at 1000 g for 10 minutes.

Preparation of chromatin. The method of Chiu et al. (1978) was used with some modifications. Nuclei were gently homogenized in 50 volumes of 0.08 M NaCl/0.02 M ethylenediaminetetracetate (EDTA)/1 mM PMSF (pH 6.3), using a Teflon-glass homogenizer driven by hand. The suspension was centrifuged at 5000 g for 10 minutes, and the pelleted chromatin was resuspended in the same buffer. This procedure of homogenization and centrifugation was repeated twice.

Chromatin fractionation. The fractionation scheme was that of Chiu et al. (1978) with some modifications. The chromatin pellet was

extracted once for 4 hours with 15 volumes of 0.35 M NaCl/1 mM Tris-HCl (pH 7.3)/1 mM PMSF. The solution was then centrifuged for 15 minutes at 20,000 g. The supernatant contained numerous nonhistone proteins. The chromatin (pellet) was suspended by homogenization in 10 volumes of 1.5 mM NaCl/0.15 mM sodium citrate/1 mM PMSF (pH 7.0), and then centrifuged at 20,000 g for 10 minutes. This was repeated once. If necessary, the chromatin may be stored at -40°C in the suspension buffer. Pelleted chromatin was resuspended by gentle homogenization in 20 volumes of 5.0 M urea/50 mM sodium phosphate buffer (pH 7.6)/1 mM PMSF. The mixture was stirred overnight and then centrifuged at 20,000 g for 30 minutes. This supernatant fluid plus the supernatant from 0.35 M NaCl extraction contained 90-95% of the chromosomal nonhistone proteins. The pellet was resuspended in 20 volumes of 2.0 M NaCl/10 mM Tris-HCl (pH 7.4)/1 mM EDTA/1 mM PMSF and extracted overnight. This was then centrifuged at 110,000 g for 36 hours. The supernatant contained mainly histone proteins, while the pellet contained residual histone proteins and proteins which are tightly bound to DNA. The residual proteins may be recovered by dissociating the pellet in a solution containing 5.0 M urea/2.5 M NaCl/50 mM Tris-HCL (pH 8.0)/1 mM PMSF and extracting overnight. The mixture was centrifuged at 110,000 g for 48 hours. The supernatant contained DNA binding proteins and the pellet contained cellular DNA with a small amount of bound residual proteins.

Preparation of samples for SDS and two-dimensional gels. All proteins to be run on gels were dialyzed against 3x distilled water containing 1 mM PMSF for a minimum of 4 hours. This was repeated once or twice depending

upon the initial salt concentration of a protein solution. The dialyzed protein solutions were then lyophilized and placed in a sample buffer. For SDS-PAGE 50-100 µg of protein were loaded per gel. The two-dimensional gels required 400-500 µg of protein.

Two-dimensional gel electrophoresis. The two-dimensional system used was that of Hirsch et al. (1978) with some modifications. Isoelectric focusing was carried out as described by Lin et al. (1976). Disposable glass Micropets of (12.6 cm × 2 mm) were cut in half and inserted into perforated rubber stoppers that fit into a disc gel electrophoresis apparatus. The bottom of each tube was sealed with "Silly Putty" (Binney and Smith, Inc.). The gels were poured up to 1 cm from the top using a 22 gauge needle and overlayered with 7 M urea instead of H_2O to help insure equal urea content in the first dimension gel. The gel solution (1 ml) contained 0.55 gm urea; 133 µl acrylamide solution (28.38% acrylamide, 1.62% N,N'-methylenebisacrylamide); 397 µl H_2O; 50 µl of 40% ampholines (pH 3.5 to 10) (LKB); 3 µl ammonium persulfate; and 1 µl N,N,N',N'-tetramethylenediamine. The sample was dissolved in 9 m urea/2% ampholines (pH 3.5-10) (LKB)/2 mM dithiothreitol and underlayered below the 7 M urea solution via a 25 µl Hamilton syringe (about 10 µl of sample may be loaded). The solution was stored in small aliquots at -80°C. An amount of 1 µl crystal violet solution was added to the top of the overlaying solution. The upper reservoir of the electrophoresis apparatus was filled with 0.01 M H_3PO_4 (anode), and the lower was filled with 0.02 N NaOH (cathode). The samples were isoelectrically focused top to bottom or anode to cathode. Isoelectric focusing was carried out in 4

steps: 30 minutes at 50 V, 30 minutes at 100 V, approximately 1 hour at 200 V (until the crystal violet dye ran off the bottom of the gel) and 30 minutes at 300 V. After isoelectric focusing was completed, the gel tubes were broken between two metal plates. The lower plate had a groove 3.5 mm wide and 1.8 mm deep. The intact gels were wetted and removed from the groove with a spatula. The first dimension pH range was determined with a Bio-Lyte miniature reference electrode.

The first dimension gels were adapted for the second dimension by immersing them for 5 minutes in 0.05 M Tris-HCl (pH 7.5), 1% SDS, at room temperature. The gels were then frozen in hexane/dry ice and thawed in 0.05 μ Tris-HCl (pH 7.5), 1% SDS. After thawing, the freezing and thawing were repeated once. The gels were then transferred to the second adaptation buffer, 5% β-mercaptoethanol. They were allowed to equilibrate for at least 5 minutes. They were then placed on the preformed slab gels for electrophoresis in the second dimension.

The second dimension of the two-dimensional gels was SDS-PAGE. The running buffer contained 0.4 M Tris-HCl (pH 8.0); 0.1% SDS; 0.2 M sodium acetate; and 0.2 M EDTA. The gel solution contained 12% acrylamide; 0.3% N,N'-methylenebisarcylamide; 0.1% SDS; 0.2 M sodium acetate; 0.4 M Tris-HCl (pH 7.5); 2 mM EDTA; 0.075% ammonium persulfate; and 0.075 N,N,N'N'-tetramethylenediamine.

The slab gel for the second dimension was formed between two Kodak projector slide cover glasses (8.3 cm × 10.2 cm × 1.2 mm). Two spacers (1 mm × 10.2 cm) glued to the edges of the glass sheet with double-coated tape. The overlapping ends of the tape were removed with a razor blade.

A second identical glass sheet was glued on top. Two large paper clips were placed on the sides. The bottom of the frame was sealed with a layer of silicone rubber spread onto a piece of paper. After 1 hour the 12% gel was poured to within a centimeter from the top and overlayered with water. After polymerization the water was removed and replaced by running buffer. The one-dimensional gel was squeezed between the glass sheets, pushed down onto the top of the 12% gel, and smoothly aligned, making sure no air bubbles were present. Some bromophenol blue crystals were placed on top of the one-dimensional gel so as to act as a dye front. The silicone was removed and the two-dimensional gels were placed in the second dimension apparatus. Electrophoresis from cathode to anode was performed at 40 ma/gel for 24 hours.

Staining and destaining of the gel was performed after electrophoresis. The glass sheets were separated with a razor blade and the gel was removed with a spatula. The gels were fixed for 1 hour in 12% trichloroacetic acid to help remove the ampholines. After this time they were placed in destaining solution (70% H_2O; 2.7% acetic acid; 27.3% ethanol), to remove trichloroacetic acid, for 30 minutes. They were then stained for 4 hours with 3.2 g Coomassie Brilliant Blue R250; 176 ml of acetic acid; 842 ml of ethanol; and 768 ml of H_2O. After staining, the gels were destained in destaining solution (see above).

Protein determination. Protein concentrations were determined by Bio-RAD protein assay using bovine gamma globulin as a protein standard.

CHAPTER III

RESULTS

Fisher 344 rat brain and liver nonhistone proteins were isolated from old and young, male and female animals. The isolated proteins were run on two-dimensional gel electrophoresis to determine if changes in the nonhistone protein complement of a rat occur with an increase in age. The advantage of this technique is that both the molecular weight and isoelectric focusing points of a protein may be ascertained once the pH ranges and molecular weight gradient of the gels are established. This was performed for the gels used in this work.

pH range of first dimension of two-dimensional gels. The pH of a first dimension gel was taken at 5 mm intervals. It can be seen from Figure 1 that the pH gradient is linear and has a range of 4.2 to 9.5. This gradient has been found to be highly reproducible. However, on overloaded gels this range does decrease.

Molecular weight determination of proteins run in the second dimension. Molecular weights of proteins run in the second dimension (SDS-PAGE) were determined through the use of various molecular weight marker proteins (Figure 2). The distance traveled of each marker protein was measured, and plotted against its known molecular weight on semi-logarithmic paper. The unknown molecular weight of a protein may be determined simply by measuring the distance traveled on the gel, finding its corresponding point on the plotted line (log of molecular weight vs.

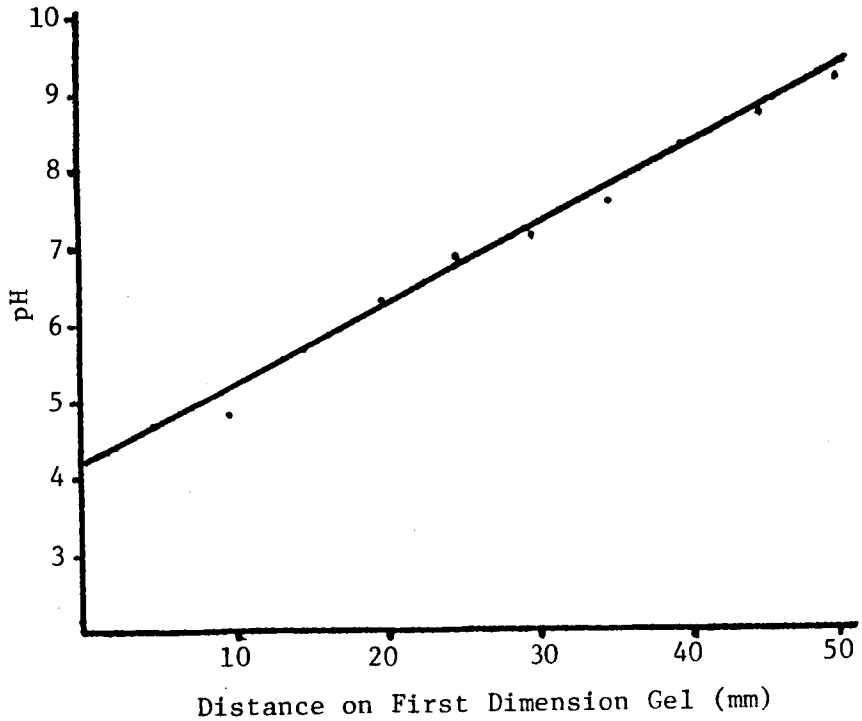


Figure 1. pH range of first dimension of two-dimensional gels. The pH range was determined through the use of a Bio-Lyte miniature reference electrode.

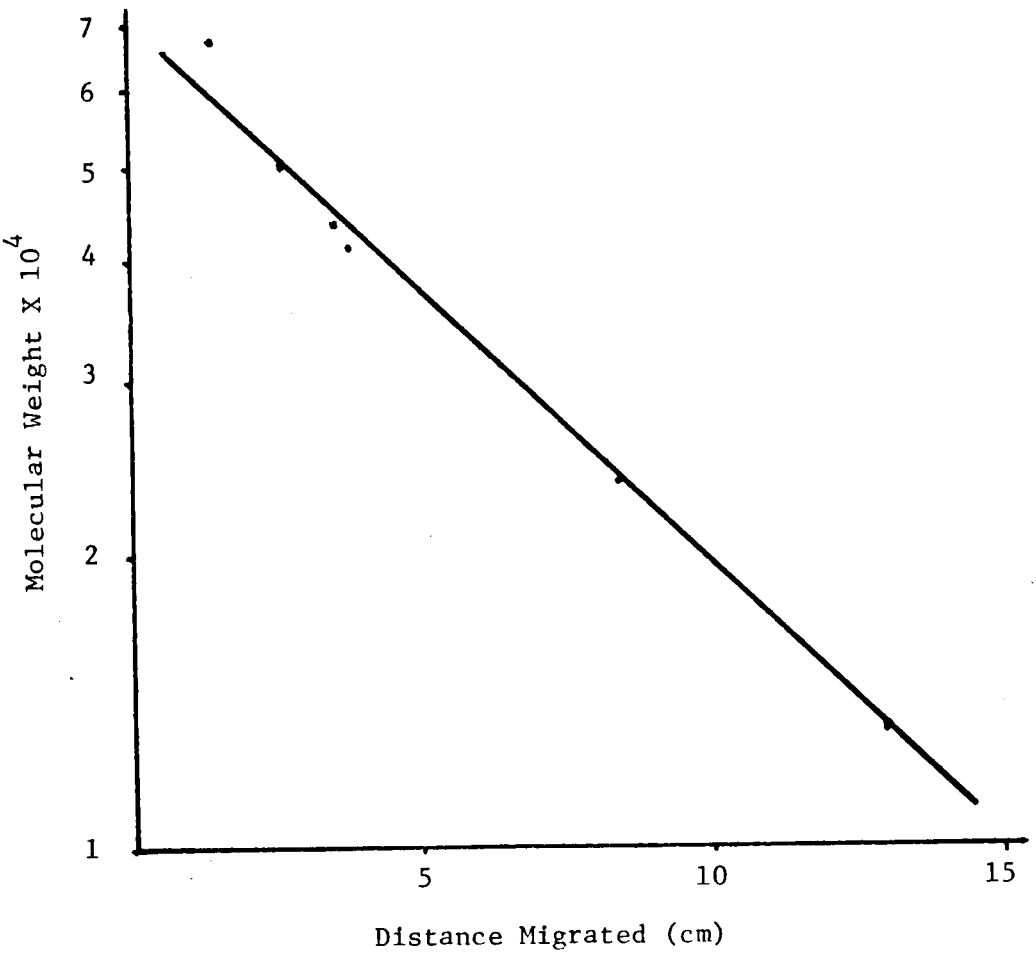


Figure 2. A semilogarithmic plot for molecular weight (MW) determination of proteins run in the second dimension of two-dimensional gels. Molecular weight markers used were: bovine serum albumin, NW of 68,000; λ globulin, MW of 50,000 and 23,500; ovalbumin, MW of alcohol dehydrogenase, MW of 41,000; and cytochrome c, MW of 13,000.

distance traveled), and then determining which molecular weight intersects the plotted line at that point.

Comparison of various NHCP isolated from old and young Fisher 344 rat brain and liver tissues. Various NHCP isolated from 4- and 29-month-old Fisher 344 rat brain and liver tissues were compared. The 0.35 M NaCl soluble and 5 M urea soluble protein fractions were compared by using two-dimensional gel electrophoresis. The 2 M NaCl soluble and the 2.5 M NaCl/5 M urea/50 mM Tris-HCl (pH 8.0) soluble chromosomal protein fractions were compared using SDS-PAGE. For both brain and liver tissues, no difference in the nonhistone protein complement was found between young and old rats. All nonhistones isolated from old and young rat brain tissues had identical protein patterns. Other nonhistones isolated from old and young rat liver tissues likewise showed no change in protein patterns associated with age.

There is, however, a difference in the nonhistone protein patterns between brain and liver tissue in the 0.35 M NaCl soluble and the 5 M urea soluble protein complements. By comparing 0.35 M NaCl and 5 M urea soluble NHCP isolated from 4-month-old Fisher 344 rat brain (Figures 3 and 4) with those isolated from rat liver (Figures 5 and 6) examples of these different protein patterns can be seen.

Comparison of NHCP isolated from male and female Fisher 344 rat brain and liver tissues. The various fractions of NHCP as described in the previous section were compared to see if there is a difference between male and female complements of NHCP. Differences in the NHCP pattern of

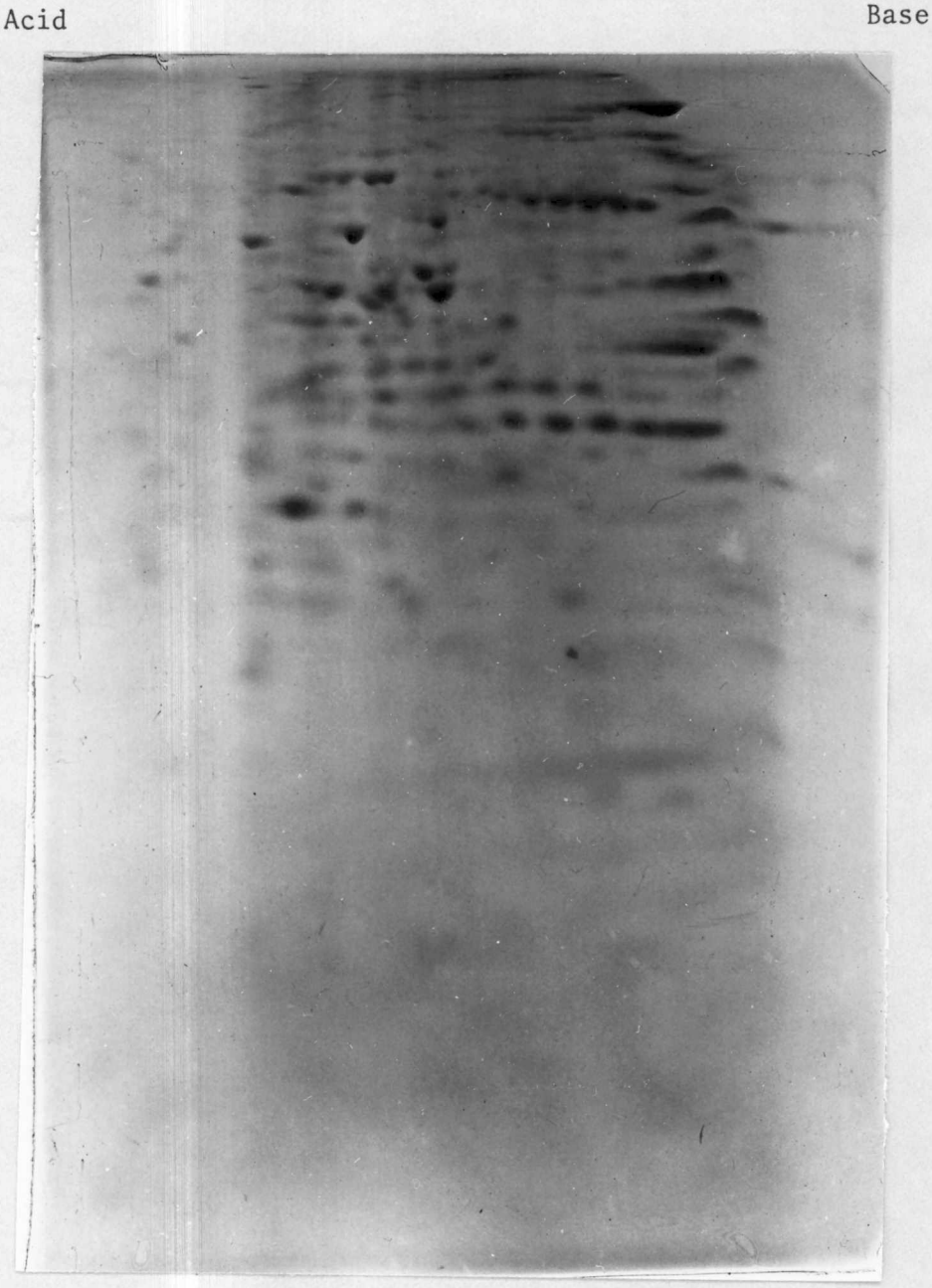


Figure 3. Two-dimensional gel electrophoresis of 0.35 M NaCl soluble NHCP isolated from 4-month-old female Fisher 344 rat brain.

Acid

Base



Figure 4. Two-dimensional gel electrophoresis of 5 M urea soluble NHCP isolated from 4-month-old female Fisher 344 rat brain.

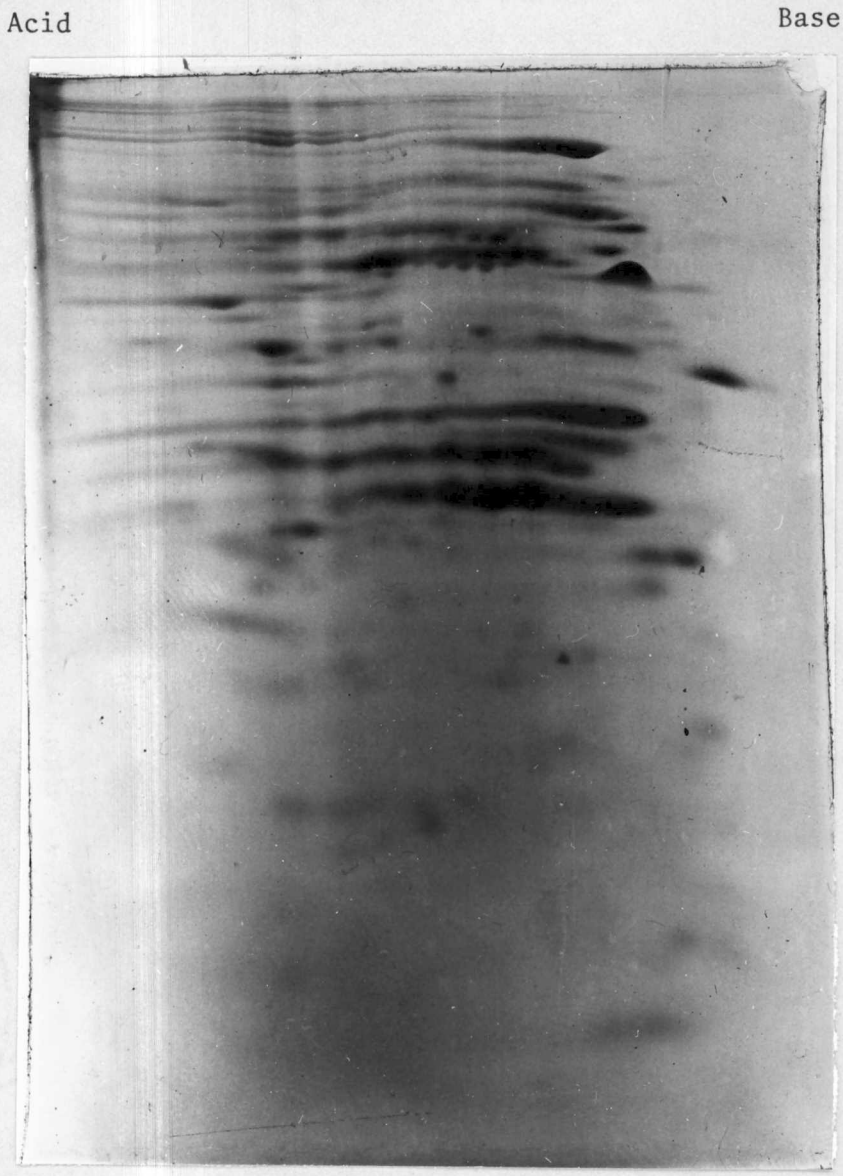


Figure 5. Two-dimensional gel electrophoresis of 0.35 M NaCl soluble NHCP isolated from 4-month-old female Fisher 344 rat liver.

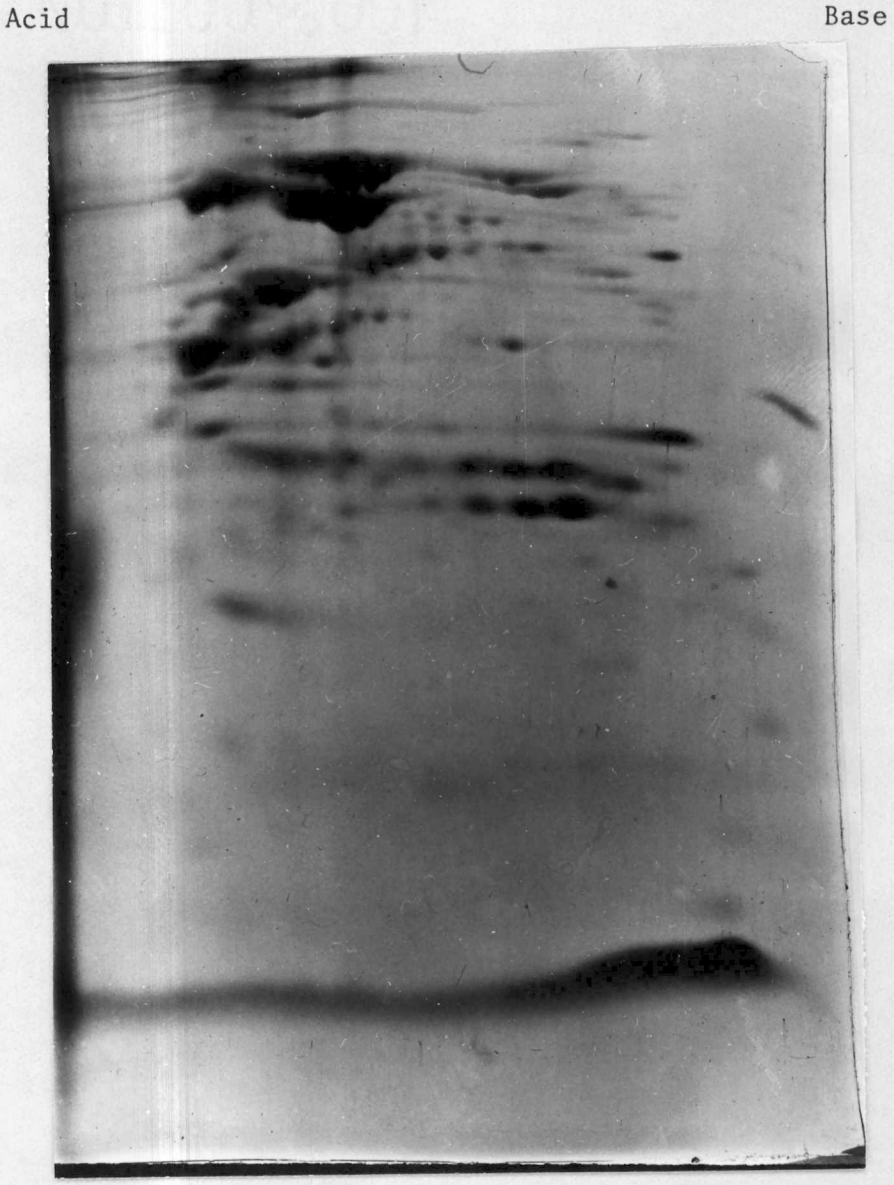


Figure 6. Two-dimensional gel electrophoresis of 5 M urea soluble NHCP isolated from 4-month-old female Fisher 344 rat liver.

two-dimensional gels were noticed between the 5 M urea soluble protein isolated from male and female brain tissue. This can be seen by comparing Figures 7 and 4. It should be noted that the male has 12 detectable proteins that the female does not. Eight of these proteins are of low molecular weight (15,000-20,000) and four are of high molecular weight (45,000-50,000). All twelve of these proteins have isoelectric focusing points of between pH 6.1 and 7. No protein differences were detected between male and female liver tissue. These results show that some nonhistone chromosomal proteins are not only tissue-specific, but also sex-specific.

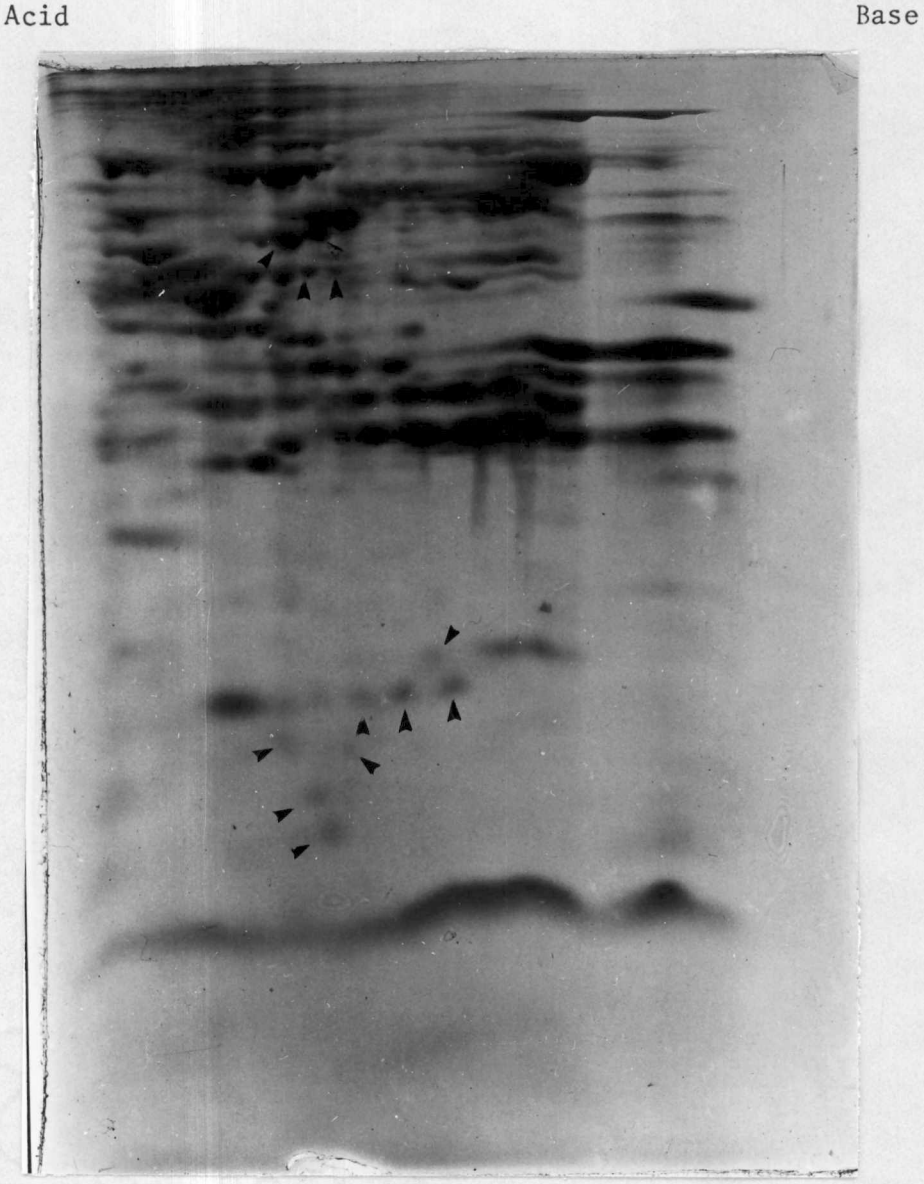


Figure 7. Two-dimensional gel electrophoresis of 5 M urea soluble NHCP isolated from 4-month-old male Fisher 344 rat brain.

CHAPTER IV

DISCUSSION

The idea that nonhistone proteins play a role in the aging process of an organism is not a new one. Many have believed for some time that the nonhistone proteins, some of which are believed to be gene regulatory proteins, may become physically altered as an organism ages. This in turn would possibly affect gene regulation so that the gene would be transcribed nonspecifically or not at all. Recent evidence (Ono and Cutler, 1978) suggests that there is an increase in nonspecific transcription as an animal ages. Medvedev et al. (1979) and Dell'Orco et al. (1978) have compared nonhistone proteins from tissues of old and young rats. The former investigators examined only the 0.35 M NaCl soluble nonhistone proteins and the comparison was made by employing SDS-PAGE. Results of this study suggest that the aging of liver cells is associated with increase in number and quantity of high-molecular weight proteins in the 0.35 M NaCl extracted fraction from the old rat liver cells. Dell'Orco et al. (1978), using cells in culture, demonstrated an apparent age-associated alteration in the incorporation of radioactive amino acids into NHCP. The age of cells is defined in this paper as whether the cells are preconfluent, confluent, or arrested in growth (G_0 phase). Both papers support the theory that aging may be related to changes in nonhistone proteins. With this in mind, the work performed in this part of this thesis was to test, via a technique with higher protein resolving capabilities, the claims that the nonhistone protein complement changes

in some way as an organism ages. The technique used was two-dimensional polyacrylamide gel electrophoresis (Hirsch, 1978). The first dimension separates proteins by their respective isoelectric focusing points, and the second dimension by the various proteins' molecular weights. This technique is much more powerful than that of SDS-PAGE used by Medvedev et al. (1979) and Dell'Orco et al. (1978), and just as reproducible. Others, like Wilson et al. (1978), have used this two-dimensional analysis to test if proteins are altered as an organism ages. They were unable to find any difference in the cellular protein complement of old and young rat superior cervical sympathetic ganglia. In this thesis, only the nonhistone proteins are analyzed. It is thought that by fractionating the nonhistone proteins into the loosely bound groups and the tightly bound groups, the resolving power of the two-dimensional gel system would be even greater.

It was decided to compare nonhistone proteins isolated from old and young rat brain and liver. Brain tissues were selected because in rats practically all types of brain cells reach final differentiation and cease to divide in the early adulthood (De Vellis and Clemente, 1970). Therefore, some cells are as old as the animal itself. Liver was chosen as a slowly dividing tissue.

After numerous comparisons of NHCP isolated from the old and young tissues, no age-related differences were found in livers or brains. This included two-dimensional analysis of the 0.35 M NaCl soluble fraction and of the 5 M urea soluble fraction. No age-related differences were found via one-dimensional analysis of the 2 M NaCl soluble proteins and

the 2.5 M NaCl/5 M urea/50 mM Tris-HCl (pH 8.0) soluble fraction. It is in agreement with the findings of Wilson et al. (1978), who found no age-related differences in total cellular proteins isolated from rat superior cervical sympathetic ganglia. The results presented in this thesis, therefore, seem to indicate that NHCP are not involved in the aging process of an organism. It should be noted that this is by no means definite. It has been estimated that in order for a protein to be detected on the two-dimensional gel system, it must be present in at least 100-500 copies per cell. The so-called gene regulatory proteins may not be present in sufficient quantity to be detected. Therefore, it still remains possible that certain NHCP, which may function as gene regulatory proteins, may become physically altered as an organism increases in age. This may only be able to be tested in the future when more sensitive techniques are developed.

It was interesting to note that an apparent tissue-specificity in certain NHCP is present between the liver and the brain. The 0.35 M NaCl and 5 M urea soluble protein fractions isolated from liver contained some proteins which were not seen in the corresponding fractions isolated from brain, and the same soluble fractions isolated from brain contained NHCP which were not found in the liver complement. This finding that some NHCP are tissue-specific is in total agreement with Miyazaki et al. (1978) and Wang et al. (1976).

One possibly significant result of these studies was that it was found that male rat brain apparently has 12 5 M urea soluble NHCP that the female rat brain does not contain. The significance of these proteins

is unknown at present, but many possibilities exist. However, it is evident that this difference in the protein complement is not due to the presence of Y chromosome in male cells, because it was found that no such difference in the NHCP complement exists when compareing male and female rat livers. Since the approximate molecular weights of the 12 NHCP are known, as well as their isoelectric focusing points, it should be relatively easy to isolate and purify them. Only after this is performed will we be able to elucidate their properties and understand their significance.

PART II

STUDIES ON NONHISTONE CHROMOSOMAL PROTEINS AND THEIR POSSIBLE ROLE IN MURINE LEUKEMIA VIRUS REPRODUCTION

CHAPTER I

INTRODUCTION

It is known that some parasitic viruses may insert their genetic information into host cell DNA. One particular example is the retroviruses which contain RNA molecules as the genomic component in the virion, and yet replicate through DNA intermediates. These viruses contain protein components which make up the virion core and membrane structures. An even more important characteristic of these viruses is that they contain the enzyme reverse transcriptase which catalyzes the synthesis of a DNA copy using viral RNA as the template. This facilitates integration of the viral genome into the host genome. This is a necessary step in retroviral replication which is discussed below.

The glycoproteins of the viral envelope facilitate adsorption and penetration of the virus into a permissive cell by interacting with host cell receptors (DeLarco and Todaro, 1976). The viral RNA is then used as a template for the synthesis of viral DNA through the action of a viral enzyme reverse transcriptase (Verma et al., 1976). There is evidence which suggests that initiation of viral DNA synthesis occurs on a tRNA primer that binds near the 5' terminus of the viral genome (Taylor, 1977). Experimental findings of "minus-strand-strong-stop DNA," terminal repeat sequences at the 5' and 3' ends of the genomic RNA molecule, "plus-strand-strong-stop DNA," and a physical map of viral DNA have allowed Gilboa and co-workers (1979) to construct a detailed scheme of biochemical reactions

for the synthesis of retroviral DNA duplexes, which contain a large repeated sequence on both ends.

On the other hand, studies in vivo have indicated that the synthesis of viral DNA in the permissive cells begins in the cytoplasm within one hour after infection (Varmus et al., 1974 and Lovinger et al., 1975) and proceeds as far as the production of linear viral DNA duplexes (form III) (Bishop, 1978). Pulse chase experiments performed with density label demonstrated that at 5-12 hours after the infection some of the duplexes turned into closed circular DNA duplexes (form I) (Shank and Varmus, 1978). That the circularization of viral DNA occurs in the nucleus is suggested by two observations: (1) all form I viral DNA is found in the nucleus and not in the cytoplasm, and (2) the linear duplexes are found in the nucleus at the same time circular DNA appears (Shank and Varmus, 1978). Although no solid evidence exists, it is believed that form I viral DNA is the immediate precursor of the integrated proviral DNA in the chromosomal DNA of the host cell. It is also believed that a circular form permits integration to occur by crossing over at a single site and results in a provirus that duplicates the linear order of the viral genome (Bishop, 1978). Regardless of whether it is the circular or linear form that integrates, it has been documented by various techniques that there is an apparent covalent likage of viral DNA to high molecular weight cellular DNA (Varmus et al., 1976, and Markham and Baluda, 1973). Circumstantial evidence indicates that integration of viral DNA is a prerequisite for viral replication (Guntaka et al., 1975). However, it alone is probably not sufficient to cause viral gene expression. The

synthesis of viral RNA occurs in the nucleus of the infected cell (Parsons et al., 1973) and this is performed by host enzymes. Two types of viral RNA are produced: viral messenger RNA, which is translated into viral proteins, and viral genomic RNA, which contains the entire viral genome. The viral proteins and the viral genomic RNA assemble in the host cytoplasm to form a viral core. In the process of the viral core budding out of the infected cell, it acquires an envelope. This envelope consists of host plasma membrane and some viral envelope proteins (Bishop, 1978).

It is apparent from the above brief review of the current knowledge that several stages of the retrovirus life cycle are not well understood. For example, two important questions which have not yet been answered are: (1) What causes circularization of form III viral DNA? and (2) By what mechanism does the viral DNA integrate into the cellular DNA? It is possible that host proteins, in particular nonhistone proteins, may play a role in these two functional steps. One reason for this is that it has recently been found that inhibition of protein synthesis in the early phase of retrovirus infection severely depresses the formation of circular viral DNA (Yang et al., 1980a). Another reason is that SV40 virus genome has been shown to take on the form of a minichromosome. This minichromosome, which resides in both the cytoplasm and nucleus of infected cells, has host histone and nonhistone proteins associated with its DNA. Finally, it has been shown by many (as mentioned previously) that nonhistone proteins have many diverse interactions with DNA. With these considerations, we have examined the possible changes in the quantity and quality of nonhistone chromatin proteins during the retrovirus infection.

CHAPTER II

MATERIALS AND METHODS

Isolation of RNA tumor virus DNA-protein complex. All isolation procedures for DNA, protein, and DNA-protein complexes were performed at 2-4°C, unless otherwise stated. NIH 3T3 cells in each 150 mm plate were washed twice with 1 mM Tris-HCl (pH 7.0) containing 0.14 M NaCl. The cells were harvested in 10 mM Tris-HCl (pH 7.4) containing 0.5 mM $MgCl_2$ and centrifuged immediately at 250 g for 10 minutes. The pellet was resuspended in 10 ml of the same medium for another 15 minutes and allowed to swell. After this time, the cells were homogenized in a tight-fitting Teflon-glass homogenizer (10 strokes). To the homogenate an equal volume of 10 mM Tris-HCl (pH 7.5)/0.5 M sucrose/0.1 M NaCl/0.1 mM PMSF was added to prevent disruption of nuclei. Nuclei were collected by centrifuging at 1000 g for 10 minutes. The supernatant (cytoplasmic fraction) was poured off, equilibrated to that of nuclear lysing buffer, and run on sucrose gradients (see below). The nuclei (pellet) were disrupted by gentle stirring for 1 hour in a solution of 10 mM Tris-HCl (pH 7.4)/0.25% Triton/0.15 M NaCl/1 mM EDTA/0.25 M sucrose/0.1 mM PMSF. This mixture was then subjected to centrifugation of 20,000 g for 15 minutes. The supernatant was poured off and run on sucrose gradients (see below) and the pellet (cellular chromatin) was subjected to differential salt extractions (see below).

Analysis of viral DNA-protein complexes. Aliquots of cytoplasmic and soluble nuclear fractions from infected NIH 3T3 mouse cells were loaded onto 5-20% sucrose gradients containing 10 mM Tris-HCl (pH 7.4)/ 50 mM NaCl/1 mM EDTA/0.5% Triton/1 ml PMSF. They were centrifuged in an SW41 rotor for 1 hour and 45 minutes at 39,000 rpm. The gradients were then fractionated into 1 ml samples. To each sample was added 50 µl of 0.2 M EDTA; 100 µl of 5 M NaCl; 100 µl of 10% SDS; and 30 µg of yeast tRNA (to serve as a carrier for precipitation of viral DNA). The samples were extracted with 1 ml of phenol and 1 ml of chloroform-isoamylalcohol in a 25: 1 ratio. The mixture was vortexed and then centrifuged at 7,000 g for 10 minutes. The top layer (aqueous phase) was removed after each centrifugation. This phase contained most of the viral DNA. Six ml of -25°C 95% ethanol was added to the aqueous phase, and the DNA was allowed to precipitate overnight. The DNA was pelleted by centrifuging for 15 minutes at 17,300 g, the ethanol was poured off and the pellet was lyophilized. To each sample of 100 µl of sample buffer was added. This buffer consisted of 10 mM Tris-HCl (pH 7.6) and 10 mM EDTA/5 mM NaCl/0.5% SDS/10% glycerol and bromophenol blue. The samples were then ready to be run on agarose gels.

Agarose gel electrophoresis and molecular hybridization. These procedures were performed as specified by Yang et al. (1980b). Hind III digested ^{32}[P] labeled λ phage DNA molecular weight markers used for aga agarose electrophoresis were obtained from W. K. Yang and C. Y. Ou.

Viral DNAs transferred to diazobenzyloxymethyl-paper were detected by molecular hybridization using virus-specific copy DNA and the dextran

sulfate procedure of Wahl et al. (1979). The viral probe used was (α-^{32}P)dCTP labeled copy DNA of Gross N ecotropic murine leukemia virus (MuLV). This was obtained from W. K. Yang, C. Y. Ou, and J. O. Kiggans.

Autoradiographs of the hybridized viral probe were made with Kodak X-ray film which was exposed at -80°C.

Differential salt extraction of cellular chromatin isolated from N-tropic murine leukemia virus infected NIH 3T3 cells. Cellular chromatin was isolated as previously described. The chromatin pellet was extracted three times; once with 0.15 M NaCl, once with 0.25 M NaCl, and once with 0.35 M NaCl (in this order for 0.5 hours each). All extraction solutions contained 0.1 mM PMSF. At the end of each extraction period, the solution was centrifuged for 15 minutes at 20,000 g. The supernatant was removed and extracted with phenol to obtain nucleic acids which were separated by electrophoresis, transferred to DBM paper and hybridized with viral probe as previously mentioned. The pellet remaining after the 0.35 M NaCl extraction was subjected to a DNA extraction procedure of Hirt (1967). The extracted DNA was separated by agarose gel electrophoresis, transferred to DBM paper and reacted with specific viral cDNA probe.

Preparation, infection, and ^{3}[H] leucine labeling of NIH 3T3 cells. NIH 3T3 cells were grown as a monolayer on 150 mm size culture dishes, using Eagle's minimal essential medium (EMEM) containing 10% fetal calf serum. Each dish initially contained 2×10^{6} cells and these cells were allowed to grow overnight. The following morning, 10 plates were infected for 1.5 hours with N-tropic virus, moi > 1 (for acutely infected cells),

and 10 plates (uninfected cells) were given plain EMEM for 1.5 hours. After this time, the cells were placed in EMEM containing 10% fetal calf serum. The next morning, all the plates, including 10 plates of chronically infected cells, were rinsed with EMEM and then incubated for 4 hours with leucine-free EMEM/10% fetal calf serum, with 250 μCi/plate (60CI/mmole) of ^{3}[H] leucine added. At the end of the incubation the ^{3}[H] leucine was rinsed off by two washes of EMEM. Then the cells were allowed to recover for 1 hour in EMEM plus 10% fetal calf serum. Upon completion of the recovering period, the cells were harvested and treated as described earlier. The cellular chromatin, which is the end product of the previous treatment, was fractionated in the same manner as tissue chromatin, except the rehydration step with 1.5 mM NaCl/0.15mM sodium citrate (pH 7.0) was omitted.

Microslab SDS-PAGE. One-dimensional SDS polyacrylamide gels were run as described by Matsudaira and Burgess (1978) with some modifications. The gel used was not a 5-20% linear gradient but instead consisted of a 6% stacking gel and a 15% separating gel. Proteins to be run on these gels were placed in sample buffer as described by Laemmli (1970). Gels were stained for 4 hours with a stock solution containing 3.2 g Coomassie Brilliant Blue R250; 176 ml of acetic acid; 842 ml of ethanol; and 768 ml of H_2O. They were destained with a stock solution containing 70% H_2O; 2.7% acetic acid; and 27.3% ethanol. Fluorography of the gels was performed by equilibrating the gels with EN^{3}HANCE (New England Nuclear), drying the gels and exposing Kodak X-ray film at -80°C. The gels were dried by the method of Matsudaira and Burgess (1978).

CHAPTER III

RESULTS

The possibility that nonhistone proteins have a function in the replication of MuLV was examined. Studies were focused on three areas. The first study was performed to determine, using sucrose gradient analysis, if viral DNA exists in the form of a minichromosome. The second study was performed to determine whether or not a relationship exists between viral DNA and NHCP. Salt extractions were used to ascertain if there is an interaction between the two. The last study was performed to determine if infection by MuLV induced or suppressed the production of host proteins, including the NHCP.

Sucrose gradient analysis of viral-DNA-protein complexes. Murine leukemia viral DNAs were isolated from nuclei and cytoplasm of infected NIH 3T3 cells. The crude preps were placed on 5-20% sucrose gradients and centrifuged. The gradients were then fractionated with each fraction being subjected to phenol-chloroform extraction. Agarose gel electrophoresis was performed for each fraction and upon completion they were transferred to DBM-paper as discussed in Materials and Methods. Figure 1 shows that in the cytoplasm only form III DNA is present and that it sediments throughout the bottom portion of the gradient. Figure 2 shows that both form III and form I viral DNAs are present in the nucleus in approximately equal quantities, with the circular forms (single and double repeat varieties) sedimenting slightly faster. Quantitation of

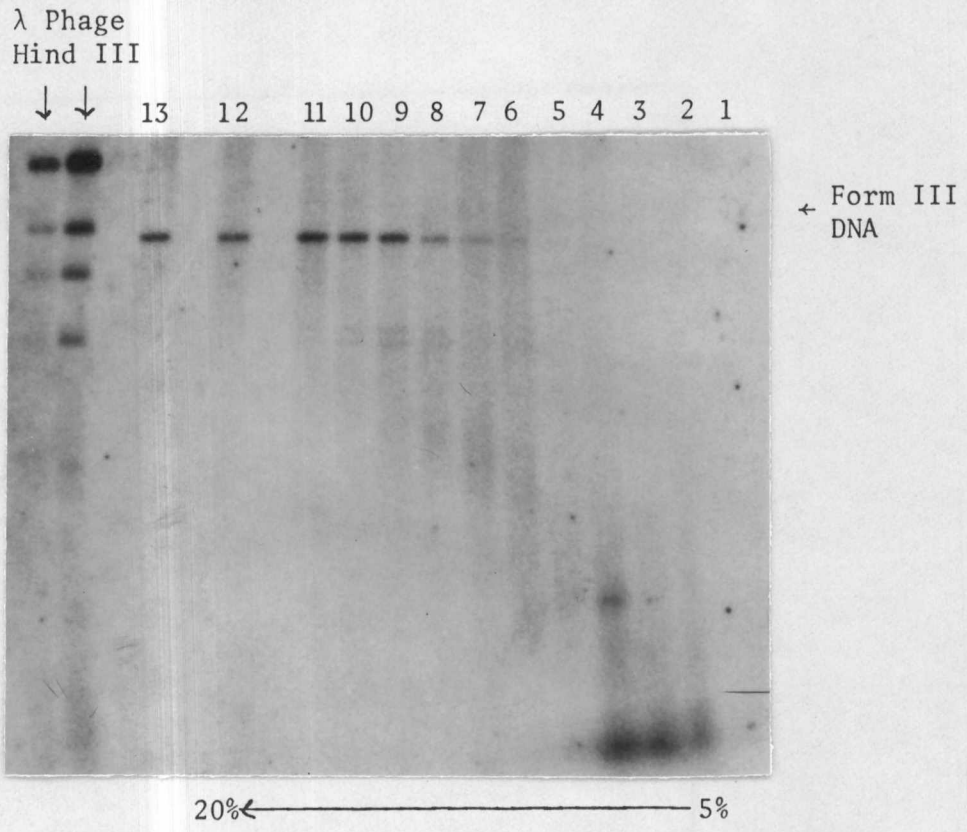


Figure 1. Autoradiograph of MuLV DNAs isolated from cytoplasm of NIH 3T3 cells. Crude preparations were placed on 5-20% sucrose gradients and centrifuged in a SW41 rotor for 1 hour and 45 minutes at 39,000 rpm. The gradients were fractionated into 13 fractions, phenol-chloroform extracted, run on agarose gels, and transferred to DBM paper. ^{32}[P] labeled viral DNA probe was hybridized to virus on DBM paper and exposed to Kodak X-ray film. Molecular weight markers are Hind III digested ^{32}[P] labeled λ phage DNAs.

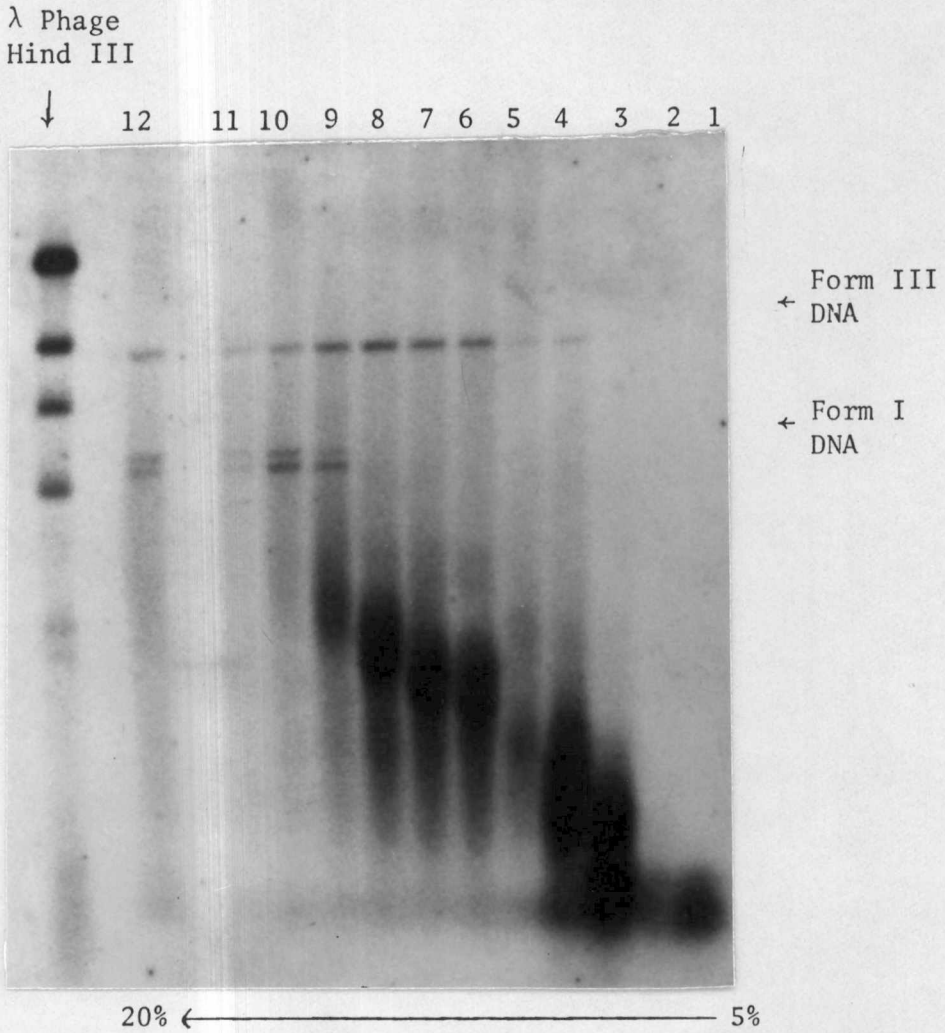


Figure 2. Autoradiograph of MuLV DNAs isolated from nuclei of NIH 3T3 cells. Crude preparations were placed on 5-20% sucrose gradients and centrifuged in a SW41 rotor for 1 hour and 45 minutes at 39,000 rpm. The gradients were fractionated into 12 fractions, phenol-chloroform extracted, run on agarose gels, and transferred to DBM paper. ^{32}[P] labeled viral DNA probe was hybridized to virus on DBM paper and exposed to Kodak X-ray film. Molecular weight markers are Hind III digested ^{32}[P] labeled λ phage DNAs.

both circular and linear forms isolated from infected cell nuclei confirms approximately equal quantities of each (unpublished results). Viral DNAs extracted by the method of Hirt et al. (1967), which presumably removes all proteins associated with the viral DNA, were also run on sucrose gradients. Figure 3 shows that these DNAs remained in the upper region of the sucrose gradient after centrifugation. It appears that viral DNA is associated with proteins in the cells, which therefore increases the rate of sedimentation.

Analysis of proteins associated with viral DNAs. In an attempt to find out what proteins are associated with the viral DNA-protein complex, sucrose gradients identical to those mentioned in the previous section were run. Upon completion of centrifugation, the gradients were fractionated as before, but this time aliquots of each fraction were run on SDS-PAGE. It was found that no correlation could be made between the fractions of the sucrose gradient where the proteins were located and the fractions where the viral DNAs were found to be located, indicating that the bulk of proteins is not associated with the viral DNA structure.

Salt extraction of viral DNAs from NIH 3T3 MuLV infected cellular chromatin. Isolated cellular chromatin from infected NIH 3T3 cells was subjected to various salt extractions. The soluble fraction of each extraction was phenol-chloroform extracted, electrophoresed and transferred to DBM-paper. In Figure 4, it can be seen that as the NaCl concentration increased, so did the amount of free form I viral DNA. Although there was still some form I DNA in the residual chromatin, as

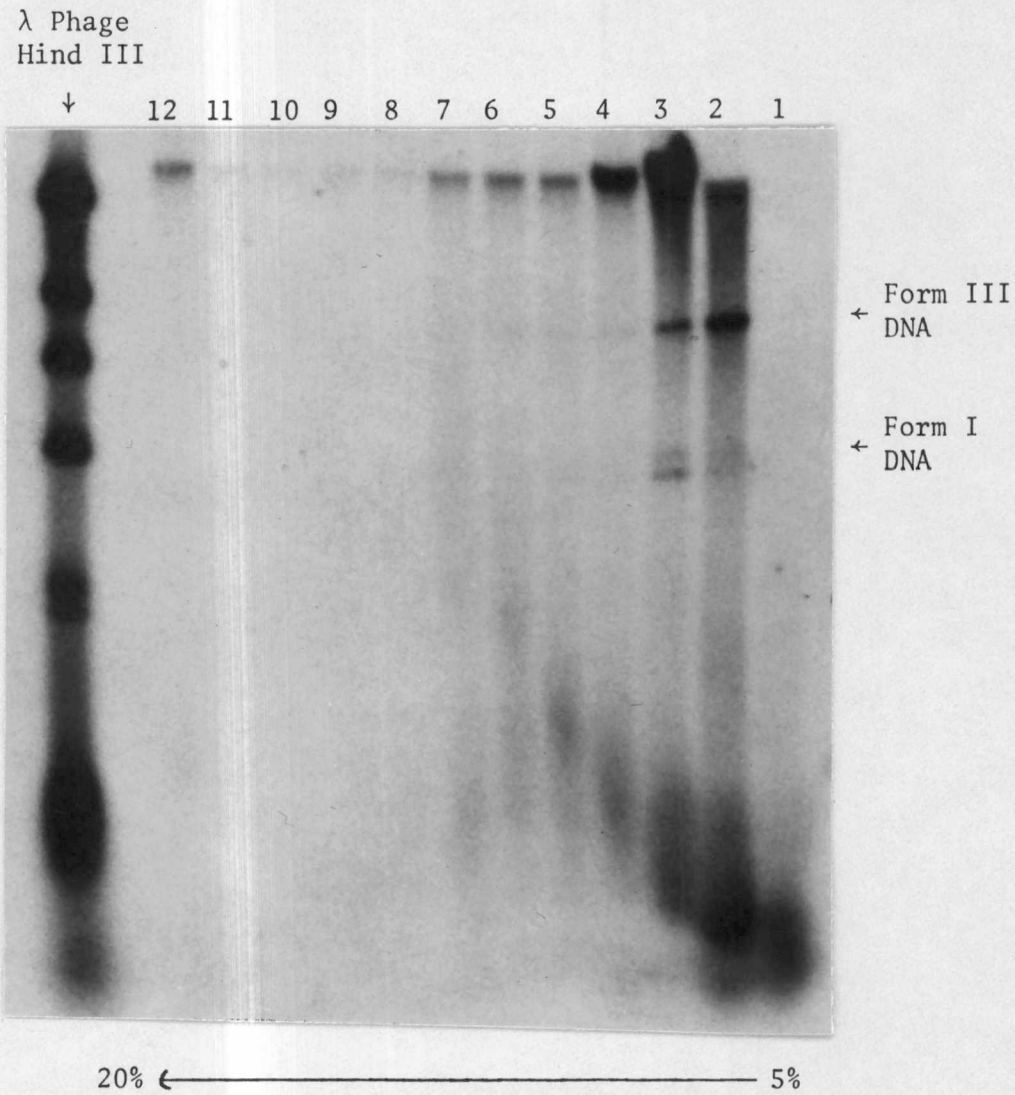


Figure 3. Autoradiograph of MuLV DNAs extracted from whole NIH 3T3 cells by the procedure of Hirt (1967). Viral DNAs were extracted from whole cells, placed on sucrose gradients and centrifuged in a SW41 rotor for 1 hour and 45 minutes at 39,000 rpm. The gradients were fractionated into 12 fractions, phenol-chloroform extracted, run on agarose gels, transferred to DBM paper, hybridized with ^{32}[P] labeled viral DNA probe, and exposed to Kodak X-ray film. Molecular weight markers were Hind III digested ^{32}[P] labeled λ phage DNAs.



Figure 4. Autoradiograph of MuLV DNAs extracted from NIH 3T3 cellular chromatin infected with MuLV. Chromatin was sequentially extracted with 0.15 M NaCl, 0.25 M NaCl, and 0.35 M NaCl. Cellular DNAs were phenol-chloroform extracted, run on agarose gels, and transferred to DBM paper. ^{32}[P] labeled viral DNA probe was hybridized to virus on DBM paper and exposed to Kodak X-ray film. Molecular weight markers were Hind III digested ^{32}[P] labeled λ phage DNAs.

can be seen after extraction according to Hirt's procedure (1967), the majority of the viral DNA became unbound with the salt extraction. The salt concentrations used here were the same as those used to remove a majority of nonhistone proteins.

Analysis of proteins isolated from uninfected NIH 3T3 cells, MuLV acutely infected cells, and MuLV chronically infected cells. Uninfected, acutely infected, and chronically infected NIH 3T3 cells were harvested and fractionated as discussed in Materials and Methods into cytoplasmic and various nuclear fractions. Acutely infected cells were infected with MuLV and grown in cultured media for 48 hours. Chronically infected cells were infected with MuLV and underwent several passages. All cells were given a pulse label of ^{3}[H] leucine as described in Materials and Methods. The various isolated protein fractions isolated from the three cell groups are discussed individually below. Differences in protein complements among the three groups of cells for each protein fraction are summarized in Table I. Some of the protein fractions were also subjected to two-dimensional gel analysis. The results are summarized in Table II.

Isolated cytoplasmic proteins from each of the three groups of cells were run on SDS-PAGE (Figure 5). It can be seen that a protein of about 41,000 MW is present in both uninfected and acutely infected cells, but is absent in the cytoplasm of chronically infected cells. This is also true for a protein with a molecular weight of 22,500. A protein of 23,000 MW is only present in chronically infected cells.

TABLE I

SDS-PAGE ANALYSIS

Protein Fraction	Protein of Interest (MW)	Coomassie Stain U[a]	A[b]	C[c]	Fluorography U	A	C
Cytoplasmic	20,000	ND[d]	ND	ND	-	-	+
	22,500	+[e]	+	-[f]	ND	ND	ND
	23,000	-	-	+	-	-	+
	30,000	ND	ND	ND	-	-	+
	41,000	+	+	-	ND	ND	ND
	47,000	ND	ND	ND	-	-	+
Soluble Nuclear	20,000	-	-	+	-	-	+
	23,000	+	+	++[g]	-	-	+
	30,000	ND	ND	ND	-	-	+
0.35 M NaCl Soluble	20,000	ND	ND	ND	-	-	+
	22,500	+	+	-	ND	ND	ND
	23,000	-	-	+	-	-	+
	26,000	+	+	-	ND	ND	ND
	30,000	ND	ND	ND	-	-	+
	33,000	+	+	-	ND	ND	ND
	34,000	-	-	+	ND	ND	ND
5M Urea Soluble	27,500	-	-	+	ND	ND	ND
	37,000	+	+	-	ND	ND	ND
	44,000	ND	ND	ND	-	-	+
2 M NaCl Soluble	none						
2.5 M NaCl 5 M Urea 50 mM Tris-HCl (pH 8.0)	none						

[a] U denotes uninfected cells.

[b] A denotes acutely infected cells.

[c] C denotes chronically infected cells.

TABLE I (continued)

[d] ND indicates no detectable differences.

[e] + indicates presence of protein.

[f] - indicates absence of protein.

[g] ++ indicates greater protein concentration than in other cell groups.

TABLE II

TWO-DIMENSIONAL GEL ANALYSIS

Protein Fraction	Protein of Interest (MW)	pH	Coomassie Stain U[a]	A[b]	C[c]
Cytoplasmic	34,000	5.6	+[d]	+	++[e]
Soluble Nuclear	none				
0.35 M NaCl Soluble	32,000	6.5	-[f]	-	+
5 M Urea Soluble	27,000	6.5	-	-	+
	54,000	5.0	++	+	+

[a] U denotes uninfected cells.

[b] A denotes acutely infected cells.

[c] C denotes chronically infected cells.

[d] + indicates presence of protein.

[e] ++ indicates greater protein concentration than in other cell groups.

[f] - indicates absence of protein.

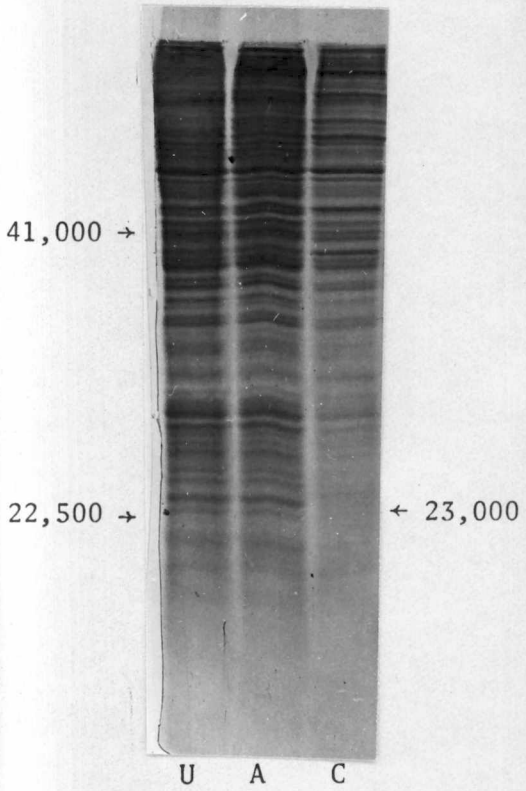


Figure 5. SDS-PAGE of the cytoplasmic protein fractions isolated from uninfected (U), acutely infected (A), and chronically infected (C) NIH 3T3 cells.

Fluorographs of similar gels (Figure 6) show that four proteins isolated from chronically infected cells incorporate ^{3}[H] leucine. These are not present in uninfected or acutely infected cells in sufficient quantities to be detected. The molecular weights of these proteins are 20,000, 23,000, 30,000, and 47,000.

Two-dimensional gel electrophoresis of these same fractions reveals only one protein difference. A protein of approximately 34,000 MW and an isoelectric focusing point at pH 5.6 appears to increase in quantity in the cytoplasm isolated from chronically infected cells (Figures 7, 8, and 9).

Nuclei from each of the three cell groups were lysed and the soluble proteins were run on SDS-PAGE. In Figure 10, it can be seen that a protein band with a molecular weight of 23,000 increases in quantity in chronically infected cells. It can also be seen in this figure that in chronically infected cells a protein of approximately 20,000 MW is present, whereas it is absent in both uninfected and acutely infected cells.

Fluographs of similar gels (Figure 11) reveal that in chronically infected cells the 20,000 and 23,000 MW protein bands incorporate a larger amount of ^{3}[H] leucine. It can also be seen that a protein of approximately 30,000 MW incorporates a large amount of ^{3}[H] leucine. Unlike the previous two proteins discussed, this protein does not show any differences in concentration, as seen on the Coomassie stained gels.

Two-dimensional electrophoresis of the various nuclear fractions reveal no differences in protein complement or protein concentrations (Figures 12, 13, and 14).

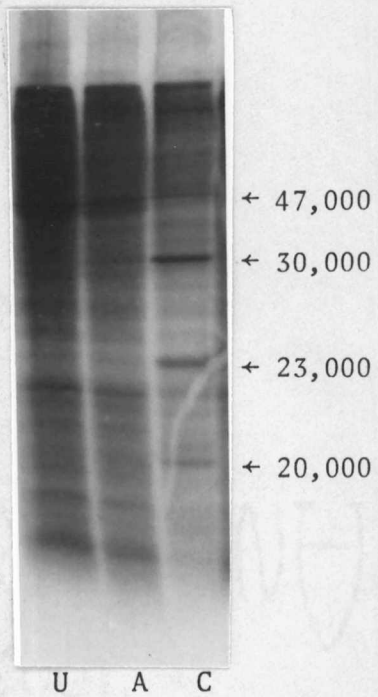


Figure 6. Fluorograph of SDS-PAGE of the cytoplasmic fractions isolated from uninfected (U), acutely infected (A), and chronically infected (C) NIH 3T3 cells.

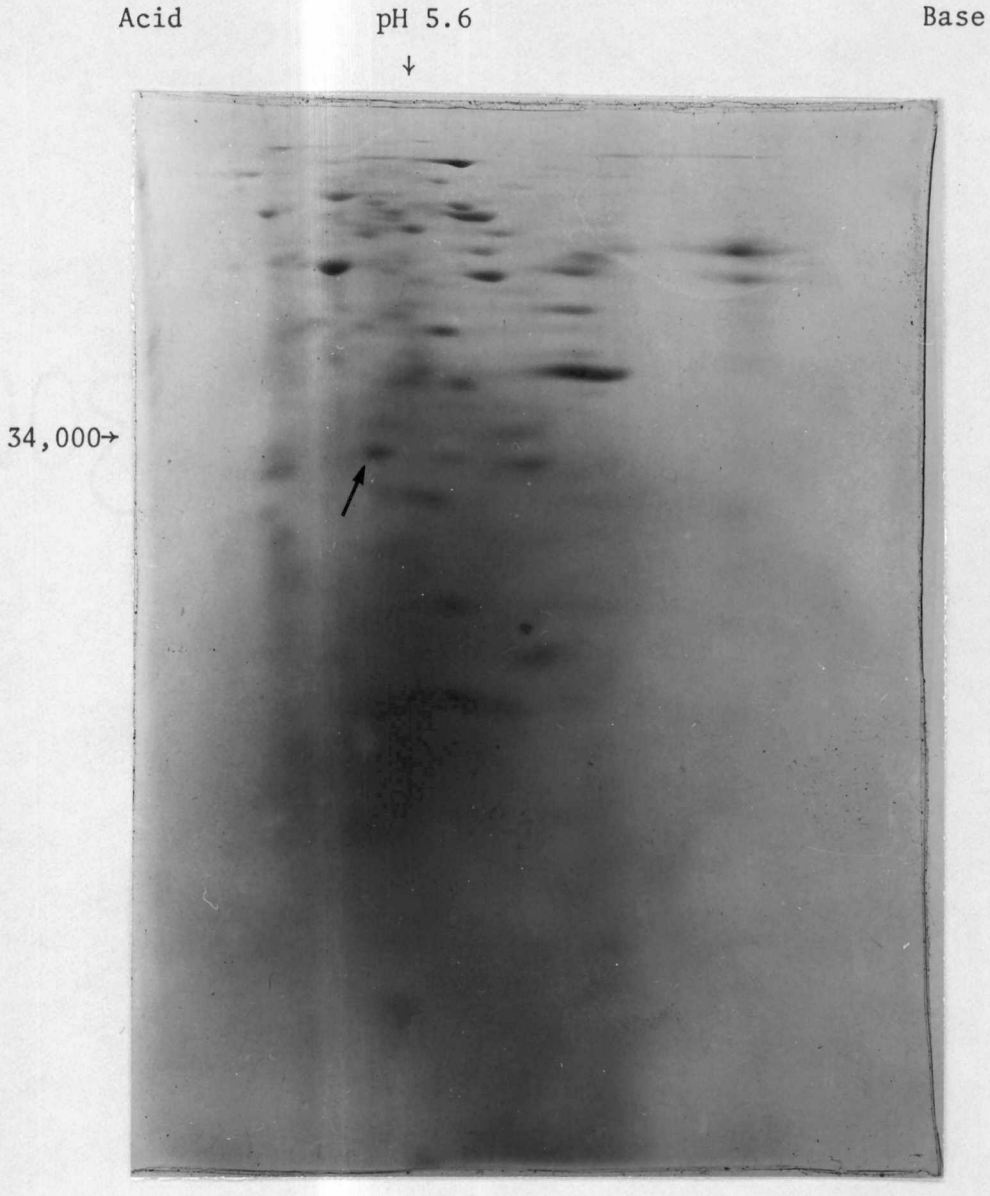


Figure 7. Two-dimensional gel electrophoresis of the cytoplasmic protein fraction isolated from uninfected NIH 3T3 cells.

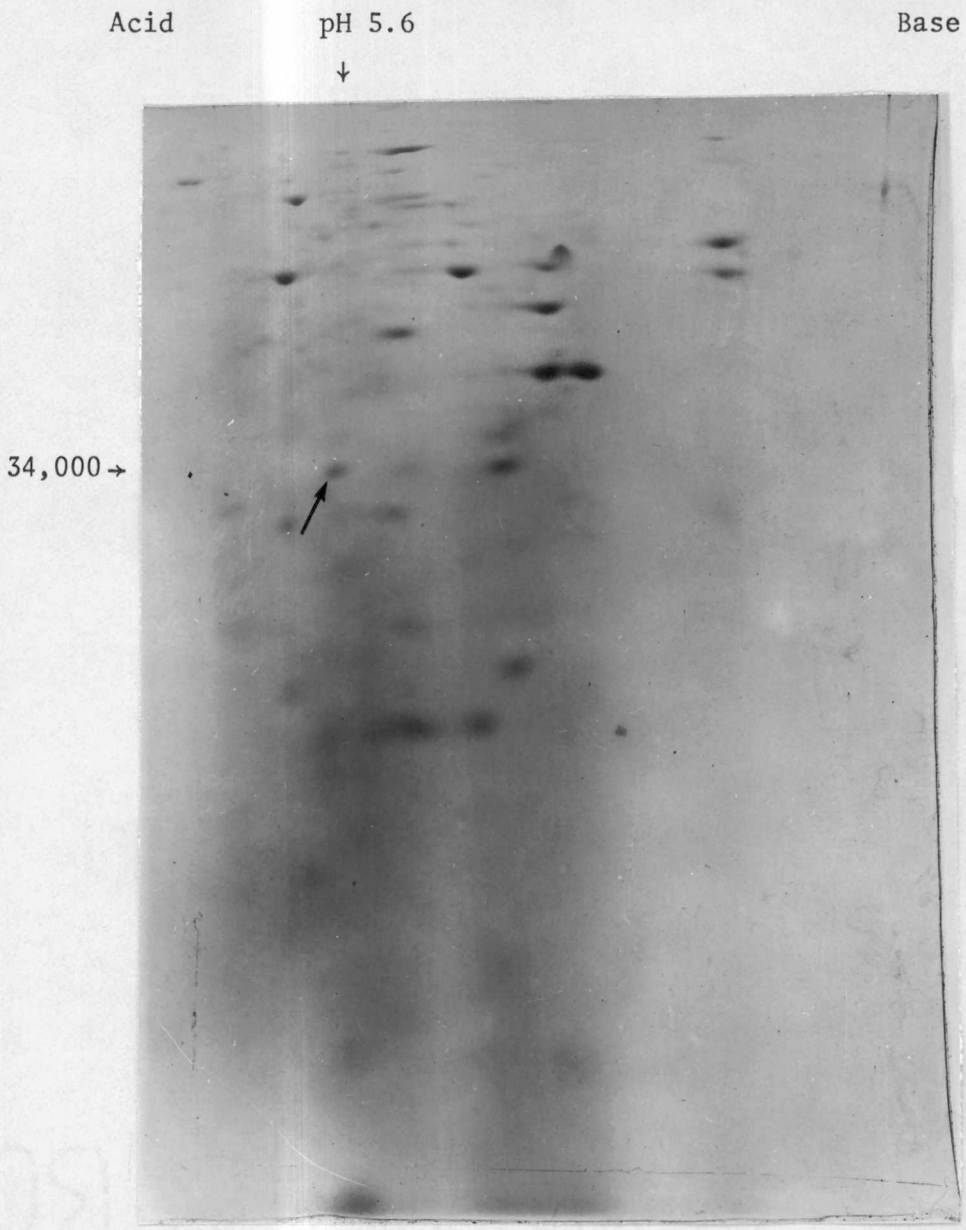


Figure 8. Two-dimensional gel electrophoresis of the cytoplasmic protein fraction isolated from acutely infected NIH 3T3 cells.

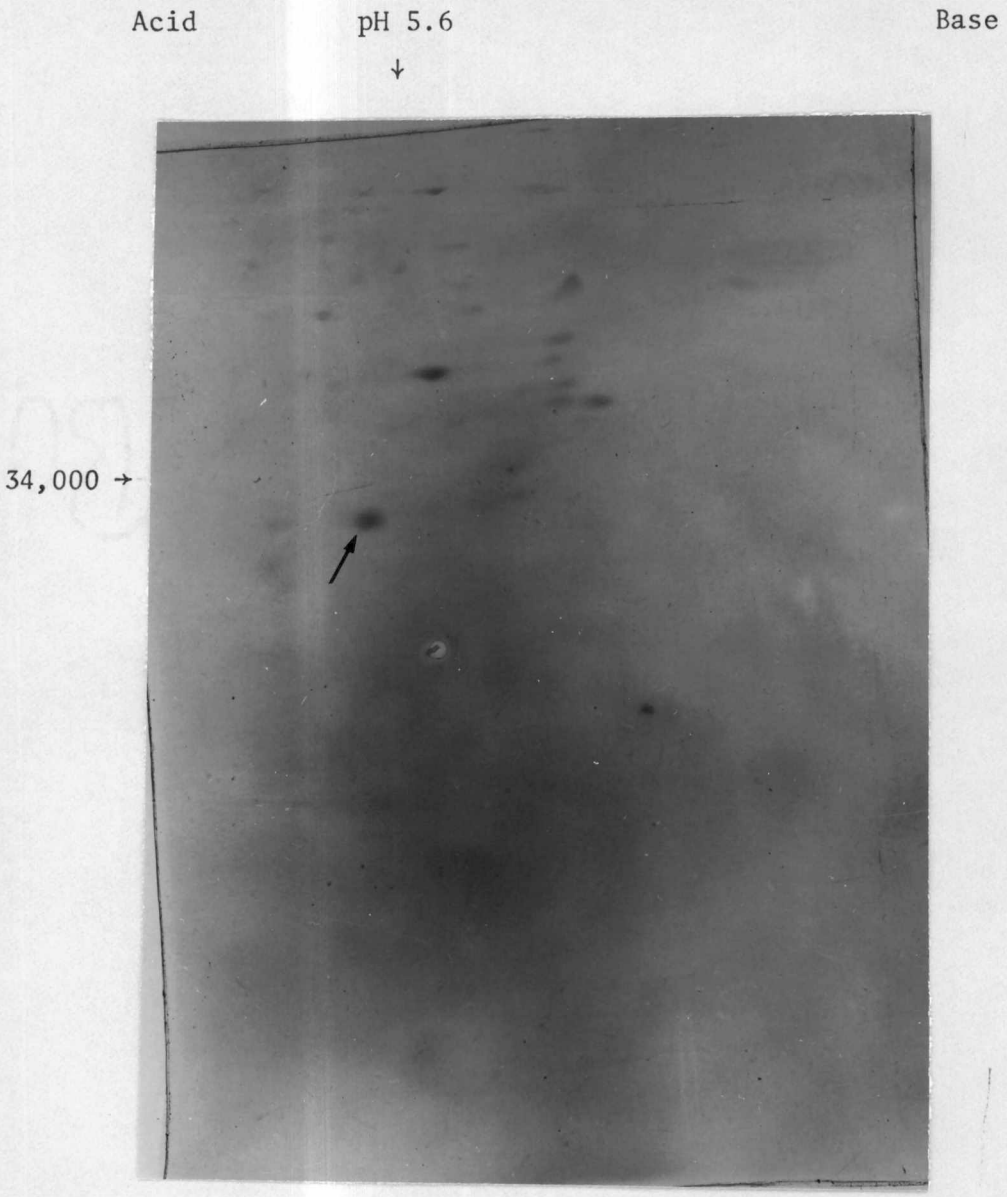


Figure 9. Two-dimensional gel electrophoresis of the cytoplasmic protein fraction isolated from chronically infected NIH 3T3 cells.

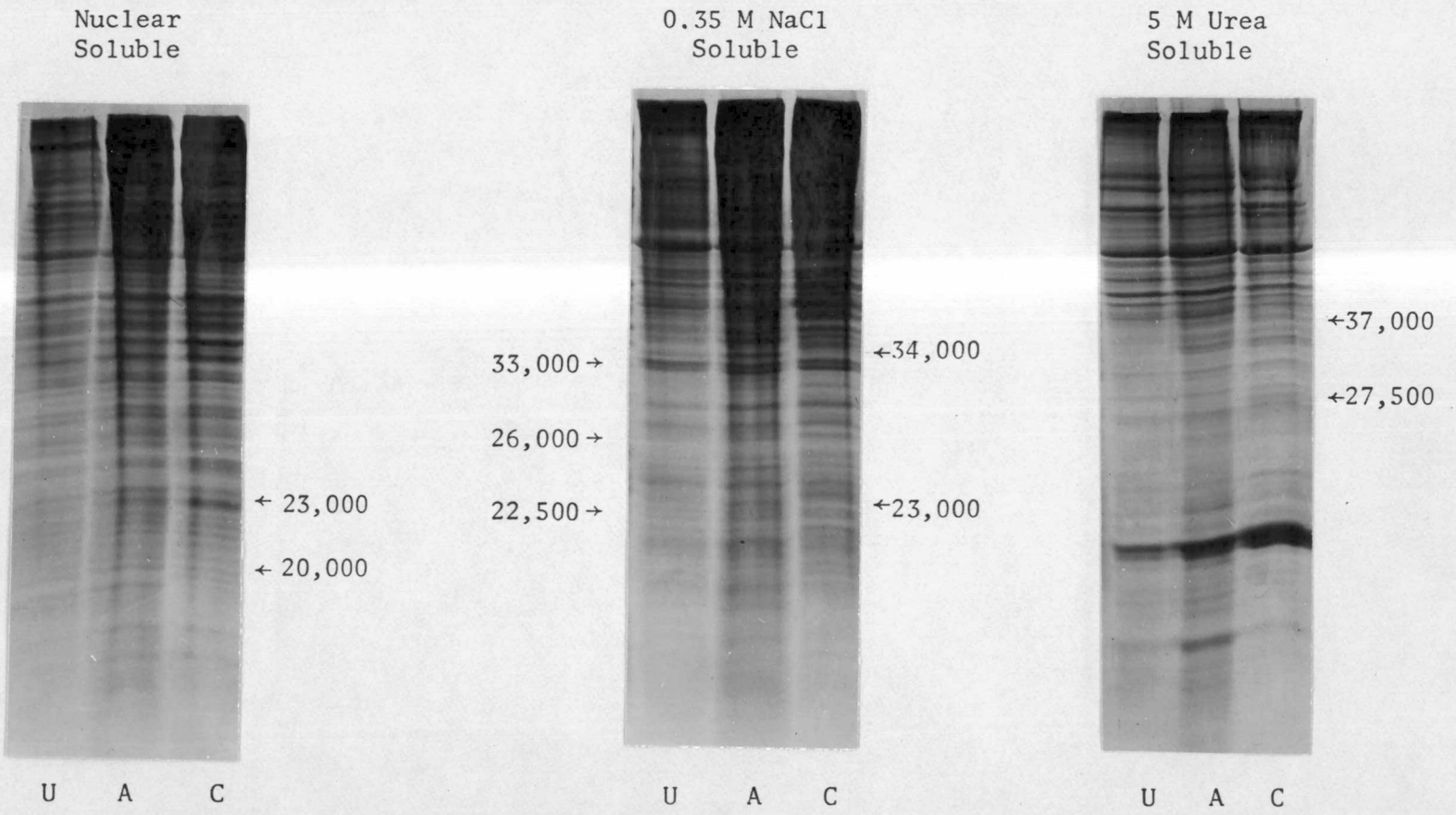


Figure 10. SDS-PAGE of the nuclear soluble, 0.35 M NaCl soluble, and the 5 M urea soluble protein fractions isolated from uninfected (U), acutely infected (A), and chronically infected (C) NIH 3T3 cells.

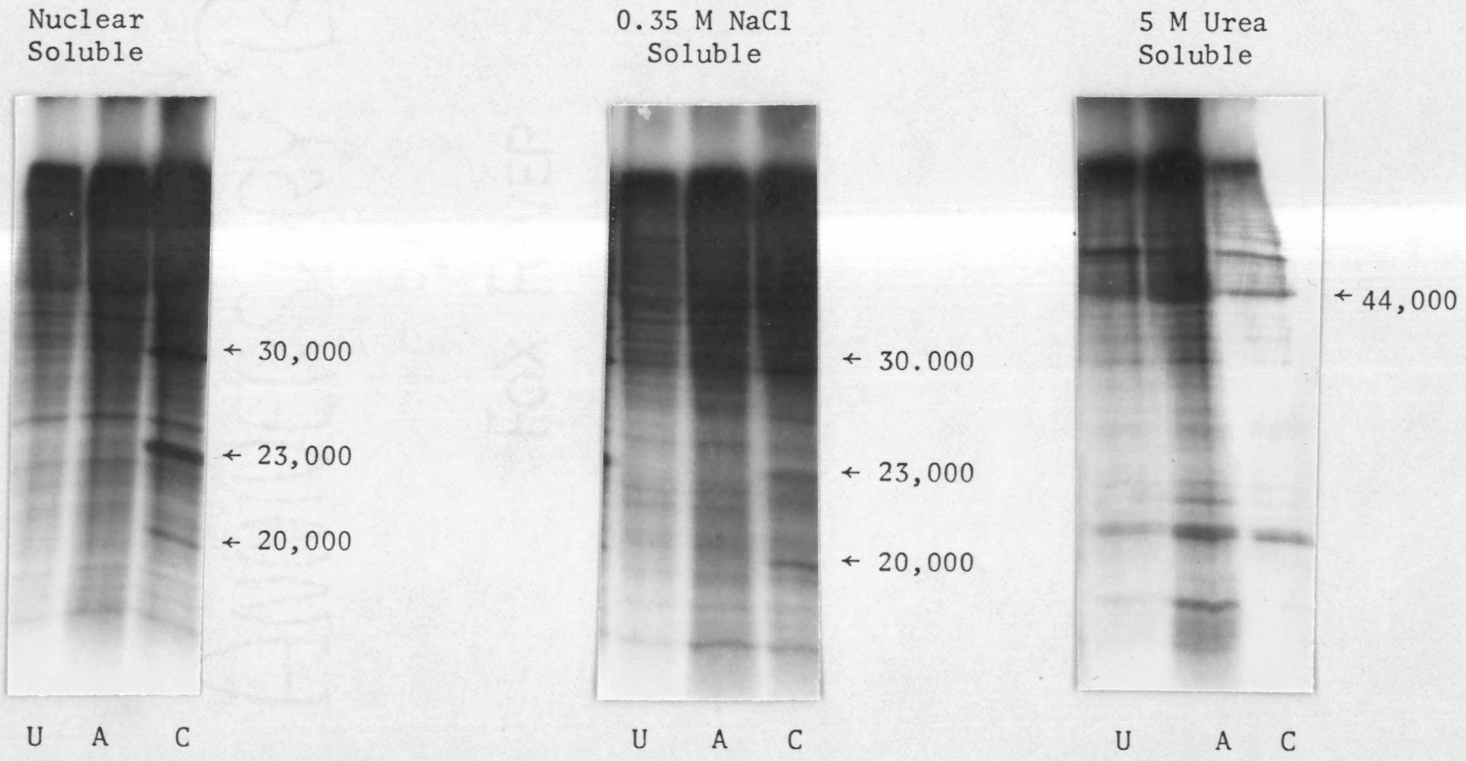


Figure 11. Fluorographs of SDS-PAGE of the nuclear soluble, 0.35 M NaCl soluble, and the 5 M urea soluble protein fractions isolated from uninfected (U), acutely infected (A), and chronically infected (C) NIH 3T3 cells.

Acid Base

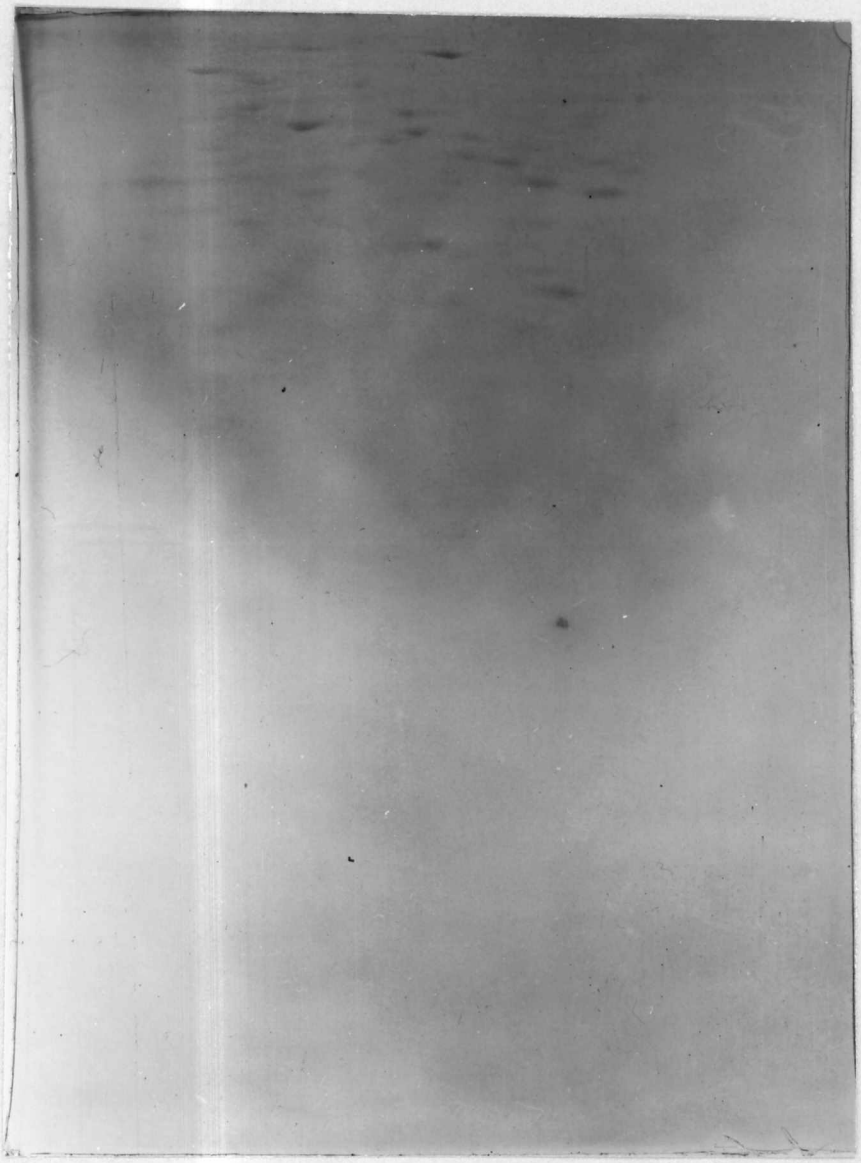


Figure 12. Two-dimensional gel electrophoresis of soluble nuclear proteins isolated from uninfected NIH 3T3 cells.

Acid Base

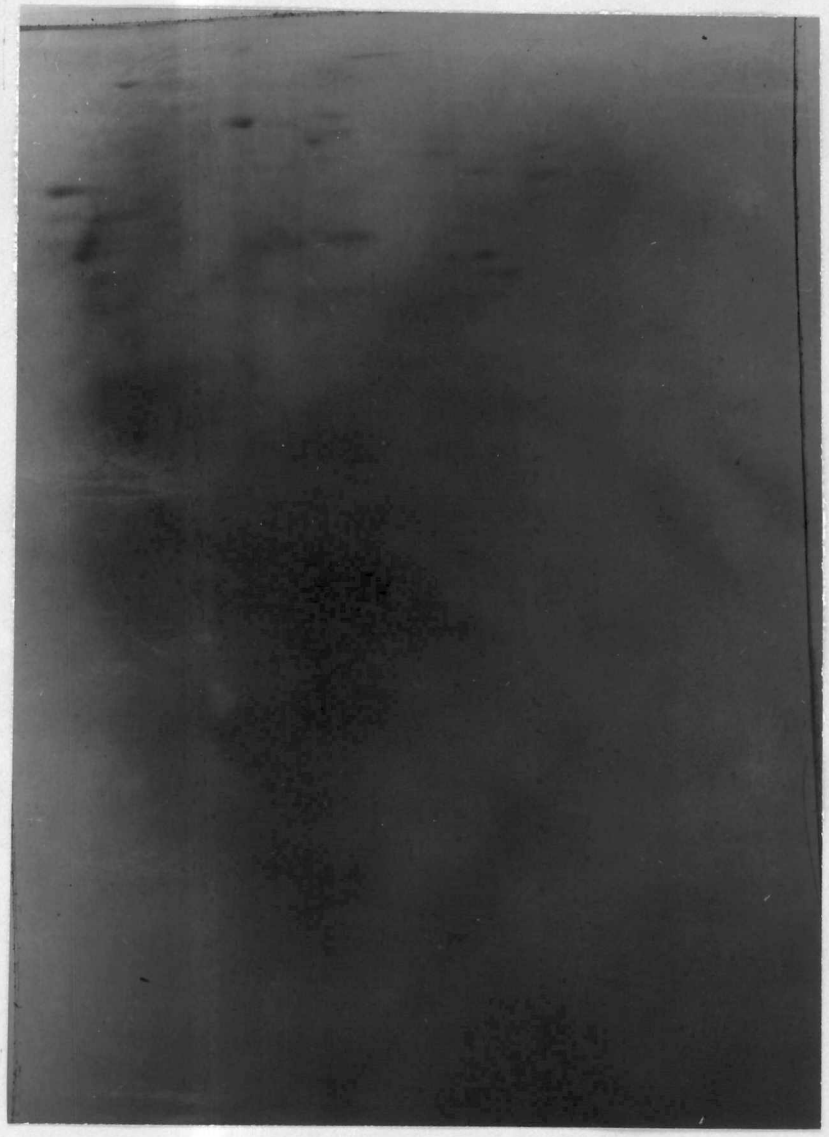


Figure 13. Two-dimensional gel electrophoresis of soluble nuclear proteins isolated from acutely infected NIH 3T3 cells.

Acid Base

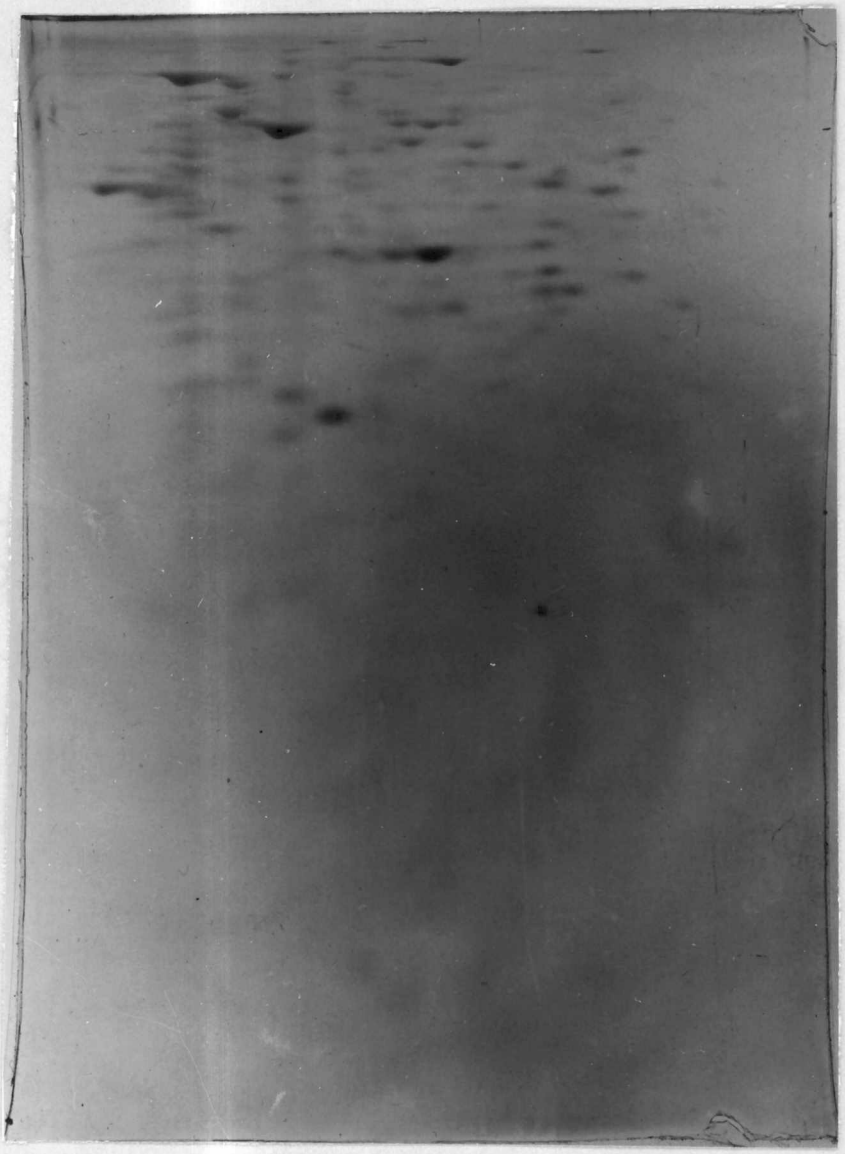


Figure 14. Two-dimensional gel electrophoresis of soluble nuclear proteins isolated from chronically infected NIH 3T3 cells.

Chromatin from uninfected, acutely infected, and chronically infected cells was extracted with 0.35 M NaCl and run on an SDS-PAGE (Figure 10). It can be seen that proteins with molecular weights of approximately 34,000 and 23,000 appear in chronically infected cells, whereas they are absent in uninfected and acutely infected cells. On the other hand, proteins with molecular weights of 33,000, 26,000, and 23,000 are present in uninfected and acutely infected cells, but are absent or diminished markedly in quantity in chronically infected cells.

Fluorographs of similar gels (Figure 11) show results identical to those received from the nuclear soluble fraction. In other words, chronically infected cells have three proteins which incorporate a comparatively large amount of ^{3}[H] leucine. These proteins are of 20,000, 23,000, and 30,000 MW.

Two-dimensional gel analysis of the 0.35M NaCl extracted proteins from the different cell groups (Figures 15, 16, and 17) reveal that one protein exists in chronically infected cells but is not present in large enough quantities to be detectable in uninfected and acutely infected cells. This protein has a molecular weight of approximately 32,000 and an isoelectric focusing point at pH 6.5.

Cellular chromatin was extracted with 5 M urea. SDS-PAGE analysis of the soluble proteins (Figure 10) shows that a protein with a molecular weight of 37,000 is present in uninfected and acutely infected cells and not in chronically infected cells. On the other hand, a protein with a molecular weight of 27,500 is present in chronically infected cells.

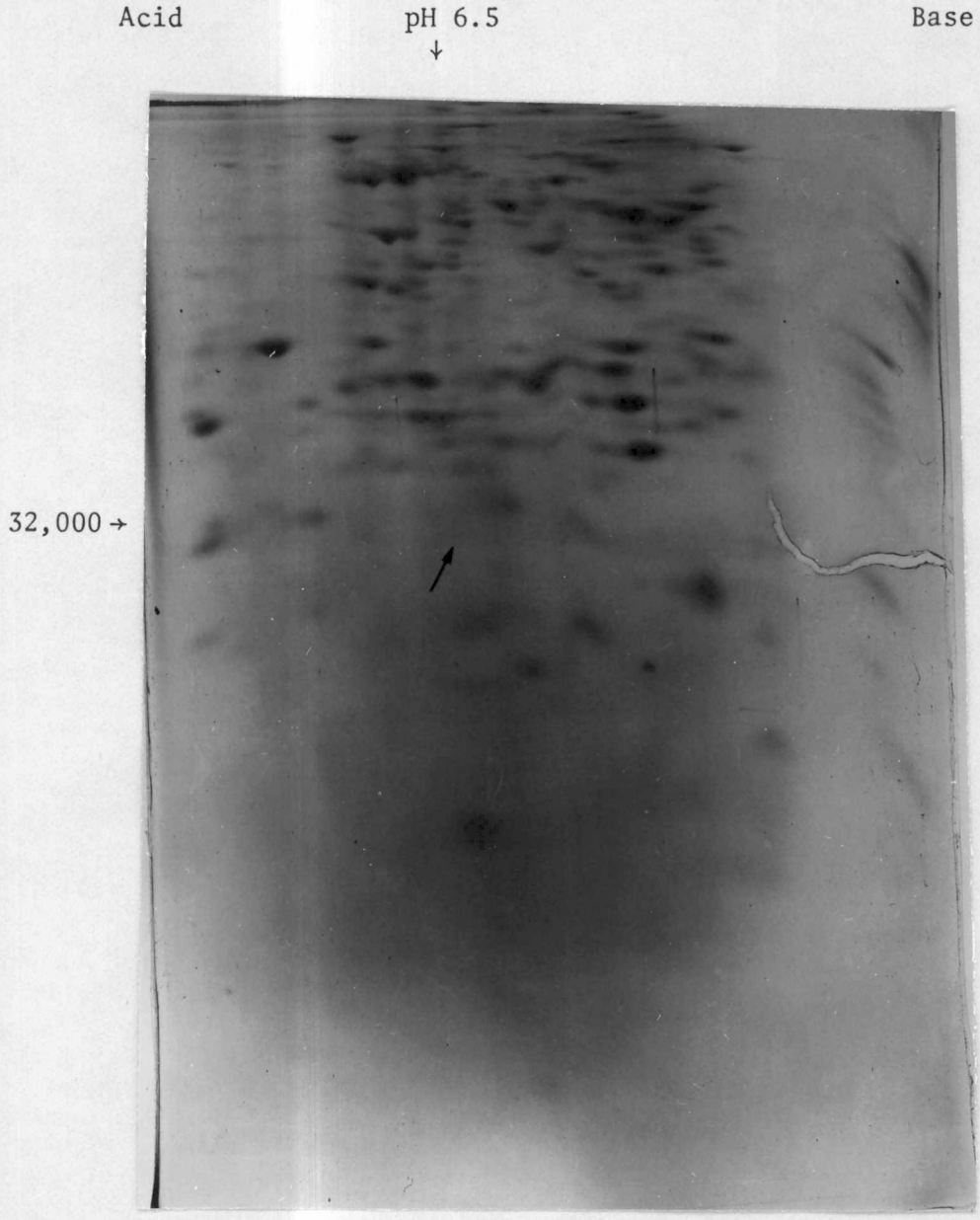


Figure 15. Two-dimensional gel electrophoresis of the 0.35 M NaCl soluble protein fraction isolated from uninfected NIH 3T3 cells.

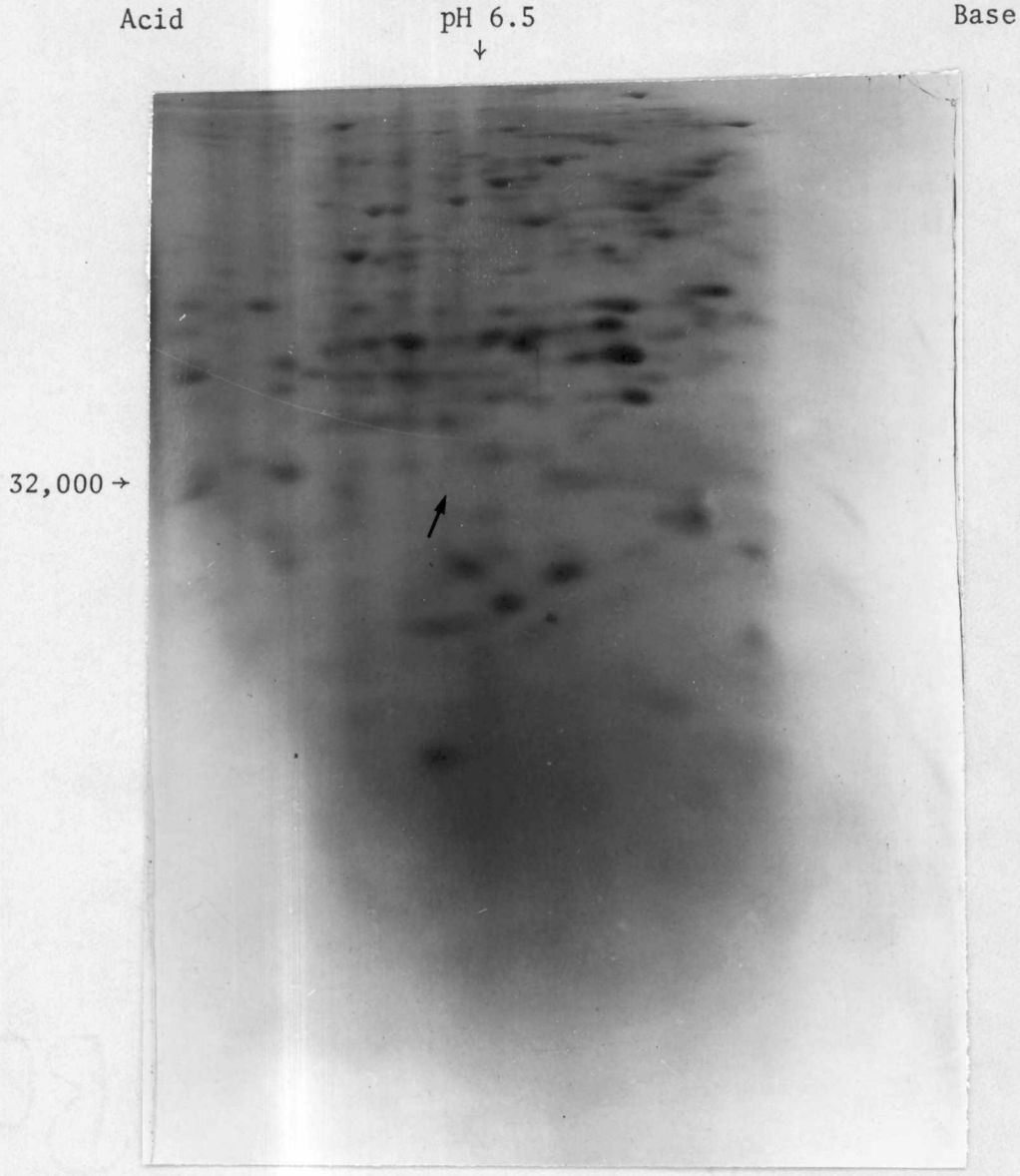


Figure 16. Two-dimensional gel electrophoresis of the 0.35 M NaCl soluble protein fraction isolated from acutely infected NIH 3T3 cells.

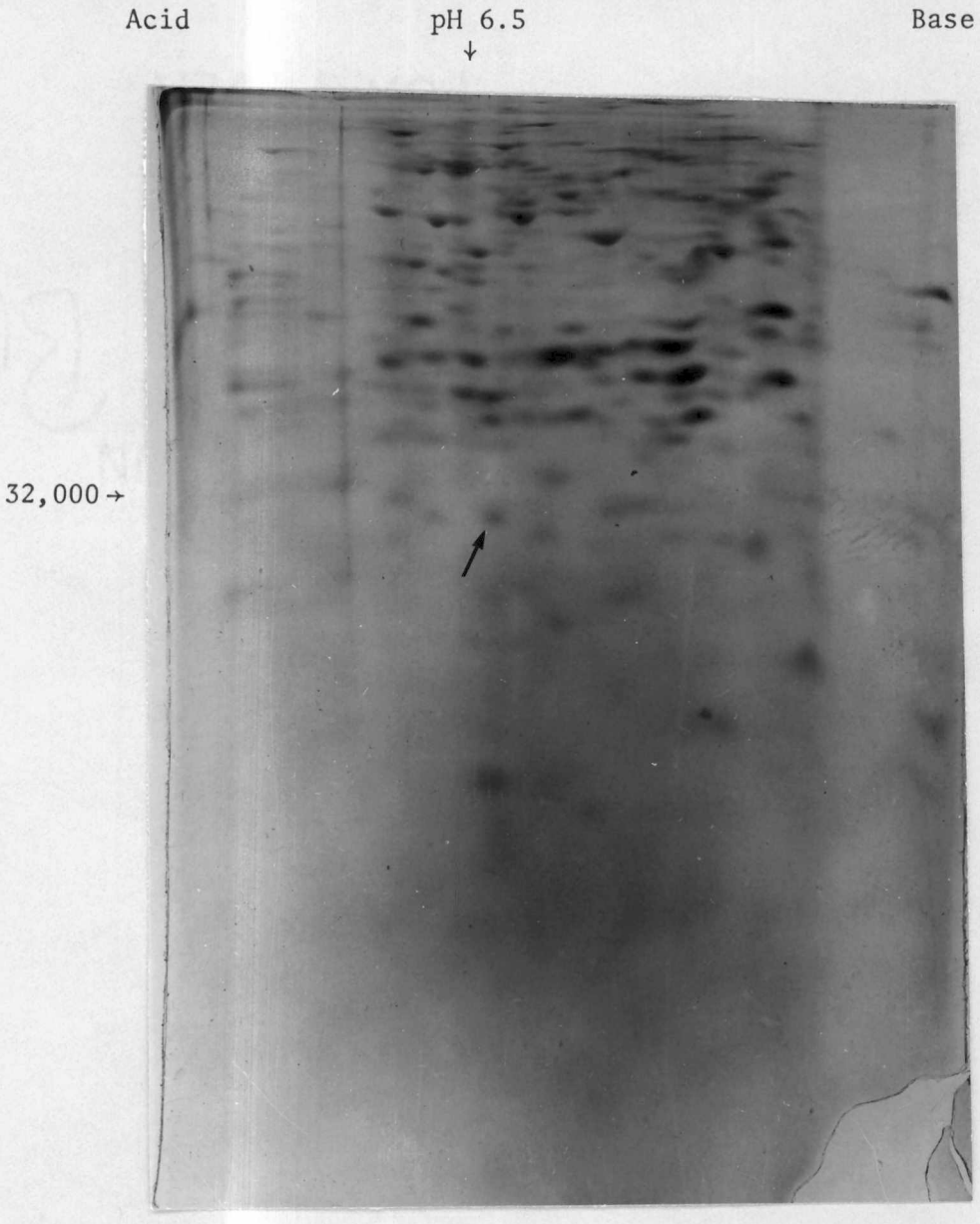


Figure 17. Two-dimensional gel electrophoresis of the 0.35 M NaCl soluble protein fraction isolated from chronically infected NIH 3T3 cells.

Fluorographs of similar gels (Figure 11) reveal that a protein with a molecular weight of 44,000 incorporates much more ^{3}[H] leucine in chronically infected cells than in the other two groups.

From two-dimensional gel analysis of these fractions (Figures 18, 19, and 20) it can be seen that in uninfected cells a protein with a molecular weight of approximately 54,000 and an isoelectric focusing point of pH 5.0 is present in larger quantities than in either of the infected cell groups. Another protein, which has a molecular weight of approximately 27,000 and an isoelectric focusing point of pH 6.5, is detectable only in chronically infected cells. This may correspond to the protein with a molecular weight of 27,500 mentioned in the SDS-PAGE staining analysis.

The residual chromatin from each of the three groups of cells was sequentially extracted with 2 M NaCl and then with a solution of 2.5 M NaCl/5 M urea/50 mM Tris-HCl (pH 8.0). After each extraction, the soluble proteins were analyzed by SDS-PAGE utilizing both Coomassie staining (Figure 21) and fluorography (Figure 22) techniques to detect any difference among the cell groups. No differences were detected.

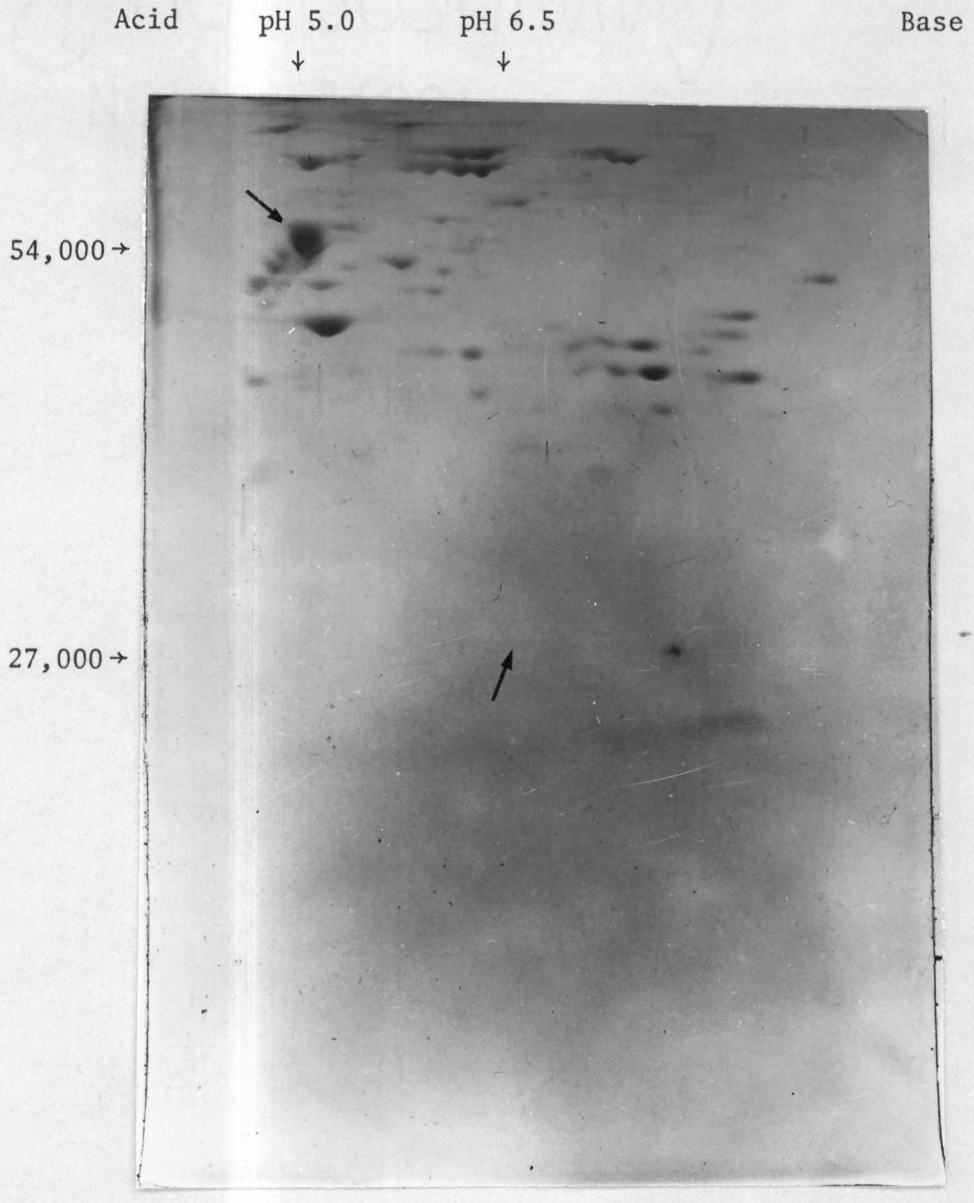


Figure 18. Two-dimensional gel electrophoresis of the 5 M urea soluble protein fraction isolated from uninfected NIH 3T3 cells.

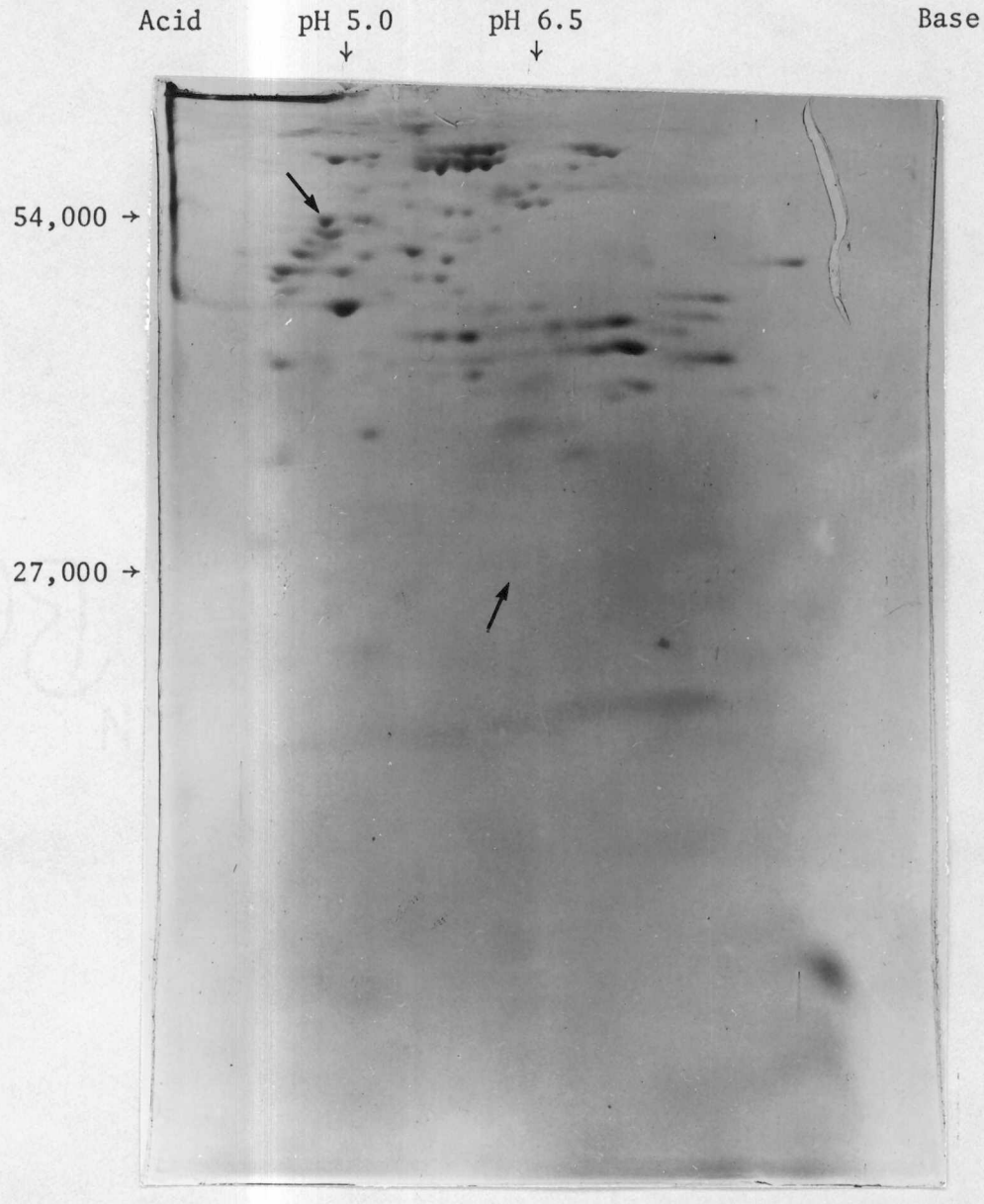


Figure 19. Two-dimensional gel electrophoresis of the 5 M urea soluble protein fraction isolated from acutely infected NIH 3T3 cells.

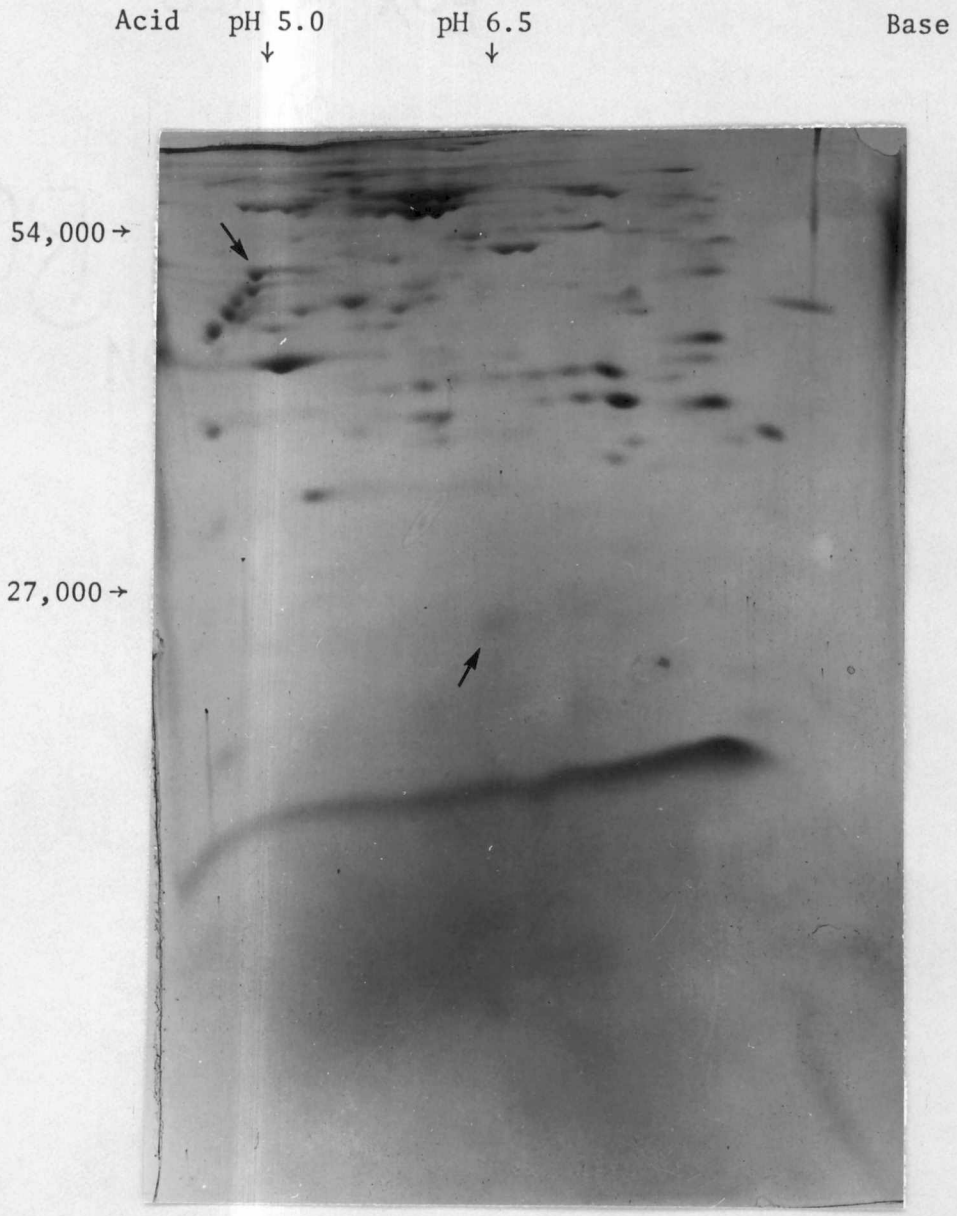


Figure 20. Two-dimensional gel electrophoresis of the 5 M urea soluble protein fraction isolated from chronically infected NIH 3T3 cells.

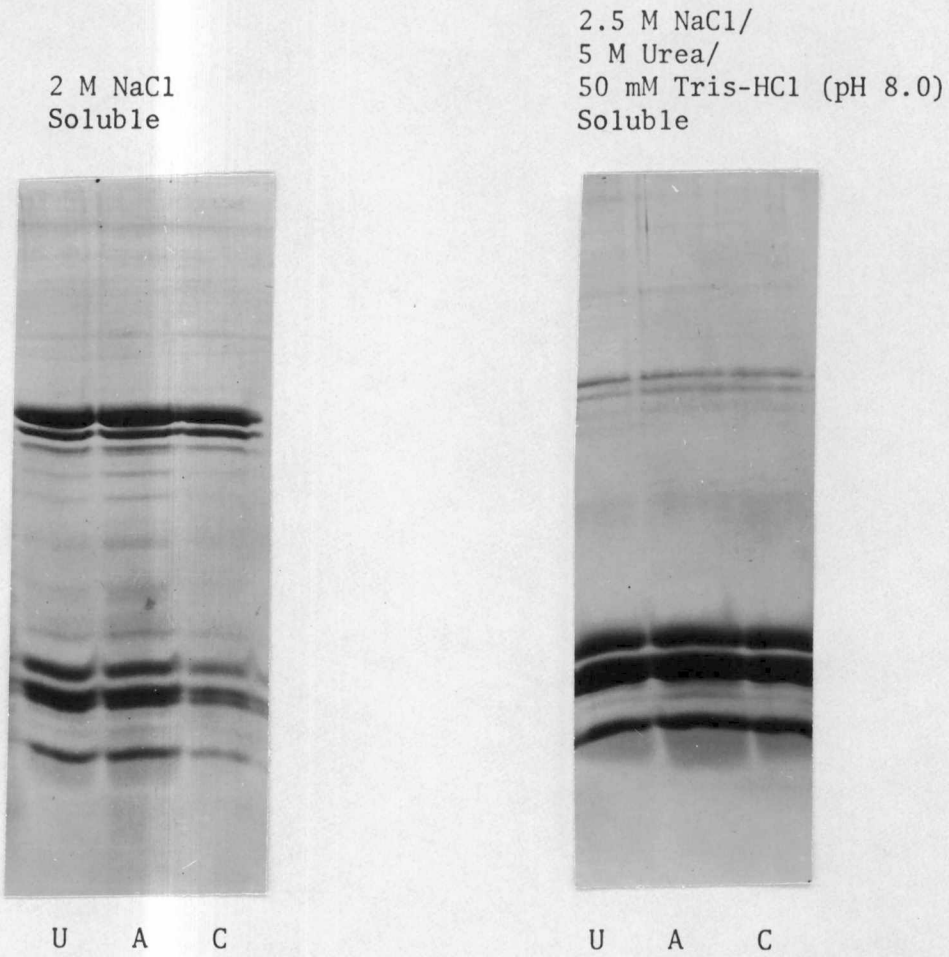


Figure 21. SDS-PAGE of the 2 M NaCl soluble and the 2.5 M NaCl/ 5 M urea/50 mM Tris-HCl (pH 8.0) soluble protein fractions isolated from uninfected (U), acutely infected (A), and chronically infected (C) NIH 3T3 cells.

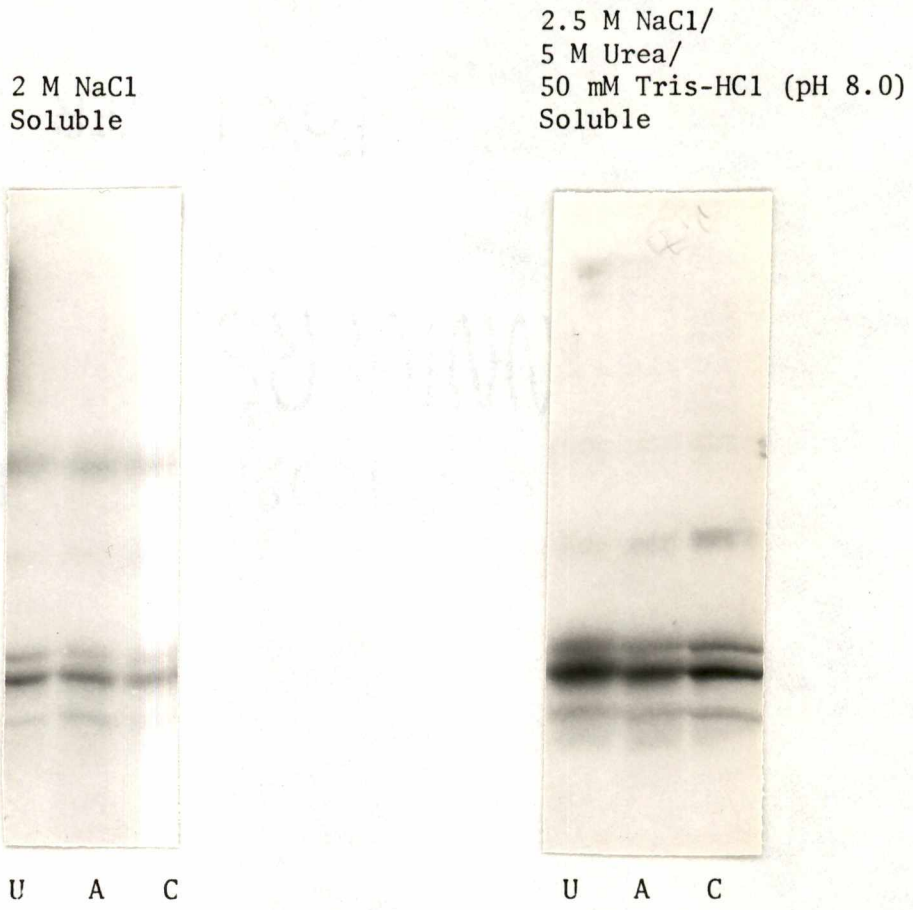


Figure 22. Fluorographs of SDS-PAGE of the 2 M NaCl soluble and the 2.5 M NaCl/5 M urea/50 mM Tris-HCl (pH 8.0) soluble protein fractions isolated from uninfected (U), acutely infected (A), and chronically infected (C) NIH 3T3 cells.

CHAPTER IV

DISCUSSION

The first group of experiments was performed in order to find evidence for the existence of a MuLV minichromosome in NIH 3T3 infected cells. It is known that some viruses exist in the forms of a minichromosome in the host cell. SV40 is a prime example of this (Varshavsky et al., 1976). The SV40 chromatin contains about 20-24 nucleosomes, all five histones, and some nonhistone proteins. It was found in sedimentation experiments presented here that forms I and III of MuLV DNA do not exist as naked DNA but instead have some material associated with them. The associated material is believed to be protein because it can be removed by the extraction procedure devised by Hirt (1967). It is interesting to note that the viral DNA-protein complex does not sediment as rapidly in a sucrose gradient as would a hypothetical MuLV minichromosome. From this it may be concluded that the MuLV DNAs do not exist in the same type of nucleoprotein complex as the minichromosome of SV40. The nature of the proteins associated with the MuLV DNA remains to be investigated.

The next experiment performed was the differential salt extraction of DNA isolated from MuLV acutely infected NIH 3T3 cells. It was found that an increasing amount of circular viral DNA was solubilized as the NaCl concentration is increased to 0.35 M. At this salt concentration, a large portion of NHCP is also removed. Thus, the results imply that one or more of the 0.35 M NaCl soluble NHCP may be associated with the viral

DNA, and this complex is in turn bound to the host chromatin via the NHCP bridge. This type of interaction logically could facilitate the integration of the viral DNA into the host DNA.

It is known that some nonhistone proteins are tissue and species-specific. It is also believed by some that integration of viral DNA is a necessary step for viral replication. From these two statements and the above, it can be hypothesized that one reason why some retroviruses may enter a cell and not replicate in it is that a specific NHCP needed for the integration process is not present in the infected cell. Because of this, the viral DNA would be unable to integrate into the host genome and therefore replication would not occur. Of course, none of this has been proven by experimental means; in fact, the hypothetical proteins may be viral in origin. However, these possibilities of NHCP-viral DNA interaction are nevertheless feasible.

In the final group of experiments, which was performed to possibly identify cellular proteins involved in the replication of MuLV, various protein fractions isolated from uninfected, acutely infected, and chronically infected NIH 3T3 cells were compared via SDS-PAGE, fluorographs of SDS-PAGE, and two-dimensional gel electrophoresis. By looking at Table I (page 44), a number of patterns can be noticed. The first one of interest is that whenever a protein difference is noticed between cell groups, the uninfected and acutely infected cells have the same result. For example: a cytoplasmic protein of 23,000 MW is only present in chronically infected cells, whereas it is absent in uninfected and acutely infected cells; likewise, a 41,000 MW cytoplasmic protein is present in

both uninfected and acutely infected cells but not in those chronically infected. The second pattern is similar to the first. This pattern is witnessed in fluorographs of SDS-PAGE in which only the chronically infected cell group contains ^{3}[H] leucine protein bands that the other two groups do not possess. In no case do the uninfected or acutely infected cells have a band that the chronically infected cells do not. It should also be noted that there is no example in which the uninfected cell group contains proteins that the acutely infected cell group does not. The reverse of this latter statement is also true. The third pattern is not as clear-cut as the first two. Upon looking at Table II (page 46), it can be seen that in two cases only chronically infected cells possess the protein, while in yet another they have an increased concentration. Only in a protein with a molecular weight of 57,000 and an isoelectric focusing point of pH 5.0 is the pattern broken. This is because only the uninfected cells display a higher concentration of this protein than is found in either of the MuLV infected cell groups. It should be noted that another possible exception exists. That exception is to the first pattern discussed above. This involves a protein with a molecular weight of 23,000. This protein is present in the soluble nuclear protein fraction and it appears that all three cell groups possess this protein, with the chronically infected cell group having a relatively higher concentration. One possible explanation of this phenomenon is that two proteins with a molecular weight of 23,000 exist in this protein fraction. One is present in all three cell groups and the other is present in only the chronically infected cell group. Unfortunately,

two-dimensional gel electrophoresis was unable to resolve this. In any case, the pattern still holds. These patterns suggest that there is not much difference between uninfected and acutely infected cells, but that there are significant differences between these cell groups and the chronically infected cells. One reason for there not being that much difference between the uninfected and acutely infected cells is that the acutely infected cells are only infected for 48 hours, therefore a small amount of the total cell population is producing mature virions. In the chronically infected cell groups, however, most of the cell population has been producing mature virions for many cell passages. The mature virions produced from one cell in this group have had time to infect a neighboring cell, so that eventually most of the cell population is producing virus products. The protein differences seen between acutely and chronically infected cell groups may be attributed to differences in the quantity of infected cells producing mature virions.

Fluorographs of various protein fractions show that some proteins isolated from chronically infected cells incorporate a great deal of ^{3}[H] leucine. Because of the labeling procedure used (described in Materials and Methods) it is unlikely that the heavy radioactivity incorporation is due to rapid turnover of proteins. It is more feasible to explain this phenomenon by considering that these proteins are, instead, being made in large quantities by a large population of infected cells. The function of these proteins is in question but it may be that they are viral proteins necessary for the completion of mature virions. Cells engaged in manufacturing large quantities of virus would incorporate

much radioactivity into the viral proteins they produce. Some proof for this last statement may exist in that MuLV is known to produce a protein with a molecular weight of approximately 30,000 ($_{p}30$). There is a protein band (shown in Table I, page 44) which shows a great deal of ^{3}[H] leucine which corresponds to a molecular weight of 30,000.

Overall, there have been many protein differences demonstrated in these experiments, yet their actual functions have not been elucidated. Some proteins have been shown not to be present in chronically infected cells but present in uninfected and acutely infected cells, whereas others are only seen in chronically infected cells. It can be speculated that in some cases the infection of a cell by a virus shuts down production of some host proteins while stimulating production of others. This may explain some of the protein differences observed in this work. It is also possible, as mentioned before, that these proteins are of a viral nature. One way to see if any proteins fit into the latter category would be to use viral-specific antibodies. In this way, viral proteins could be isolated and identified. Many other techniques could be used to discover the significance of these suppressed and induced proteins.

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VITA

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