ABSTRACT

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Recent observations in several cell lines are consistent with the hypothesis that non-histone chromatin (NHC) proteins may be involved in the regulation of the differentiation process. Significant differences have been observed in populations of NHC proteins derived from cells prior to and after the onset of differentiation. This study utilizes the 3T3-L1 preadipocyte as a model system for looking at changes in NHC proteins during differentiation. NHC proteins were extracted and characterized using SDS-polyacrylamide gel electrophoresis. The characterization of NHC proteins obtained from undifferentiated and differentiated cells show that a variety of changes in the population of NHC proteins occurs durring differentiation. Differentiation in these cells is accompanied by the appearance of proteins with molecular weights of 28,000, 30,000, 125,000, 150,000, and 200,000. A protein of 53,000 daltons was observed to vary in content with respect to time. Autoradiographic analysis of NHC proteins by densitometer scanning revealed that 11 proteins increase and 6 proteins decrease in amount when cells are induced to differentiate.

SURVEY OF NON-HISTONE CHROMATIN PROTEINS IN A DIFFERENTIATING CELL LINE: THE 3T3-L1 PREADIPOCYTE

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SURVEY OF NON-HISTONE CHROMATIN PROTEINS

IN A DIFFERENTIATING CELL LINE:

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ABBREVIATIONS

BSA	Bovine serum albumin
cAMP	Adenosine 3',5'-cyclic monophosphate
DEX	Dexamethasone
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetetraacetate
HMG	High mobility group
MIX	1-methyl-3-isobutylxanthine
m.w.	Molecular Weight
NHC	Nonhistone Chromatin
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl floride
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-tetramethylethylenediamine

INTRODUCTION

The discovery of DNA and its role in storing genetic information has led to what is considered a central tenet of modern cell biology: each somatic cell of a given eukaryote has the identical content of DNA and this is normally double the content found in gametes (Mirsky and Ris, 1952). In eukaryotic systems, current data supports the hypothesis that the content of genetic material found in the somatic cells of an organism does not normally vary (Bedi and Goldstein, 1976). Further investigation of the DNA in eukaryotic cells has raised two questions: how are such large amounts of DNA packaged into each cell, and how are specific genes expressed to determine the morphological and biochemical characteristics that distinguish cell types. The first question can be addressed by considering the organization of DNA within the nucleus.

In eukaryotes the DNA is not arranged in a single duplex molecule but is complexed with proteins to form chromatin. At the first level of chromatin organization, DNA is associated with small basic proteins called histones. These histones, with the exception of histone H1, form an octamer which is wrapped by 140 base pairs of DNA to form the core particle of the nucleosome. Found between the core particles is a linker portion of DNA which is associated with histone H1 to complete the nucleosome. Supercoiling occurs between the core particles to further condense the DNA resulting in a 10nm chain of nucleosome (Igo-Kemenes et al., 1982). The nucleosomal chain is folded in the interphase nucleus to form a solenoidal structure containing about six

nucleosomes per turn (Igo-Kemenes et al., 1982). This arrangement of DNA results in as much of a 10^4 reduction in length (Cartwright et al., 1982). The description of chromatin presented thus far begins to explain how large amounts of DNA can be packaged within the nucleus but does not explain how selected portions of this genetic material are expressed. Further examination of chromatin composition reveals possible mechanisms by which gene expression is regulated.

Because the histone proteins occur in small numbers that, with the exception of histone H1, form the core of the nucleosome and the fact that this group as a whole is evolutionarily highly conserved in composition suggests that their role is limited to providing structural organization for the chromatin material (Lewin, 1980). Also associated with chromatin is a group of proteins collectively called the non-histone chromatin proteins (NHC). The NHC proteins are defined as the "protein components in chromatin remaining after the five histones have been removed" (Lewin, 1980). The total amount of NHC proteins reported varies but a typical value is between 60 and 70% of the histone content (Lewin, 1980). This corresponds to roughly 700,000 daltons of protein for every 1,000,000 daltons of DNA (Lewin, 1980). The NHC proteins are believed to be composed of three groups that function within chromosomes: the first group includes structural proteins that compose the scaffold of chromosomes, the second group is composed of the enzymes involved in the synthesis of nucleic acids, and the third group is composed of possible regulatory proteins which control gene expression (Bradbury, 1982). This study is concerned with the NHC proteins that may play a role in gene regulation.

Recent observations in several cell lines are consistant with the hypothesis that NHC proteins may be involved in the regulation of the differentiation process (Keppel et al., 1977; Rodriguez et al., 1979; Perle and Neuman, 1980; Heizmann et al., 1980; LeParco et al., 1981; Morioka et al., 1981; Perle et al., 1982). Significant differences have been observed in populations fo NHC proteins derived from cells prior to and after the onset of differentiation.

The possible role of proteins in the regulation of gene expression was first realized through work with prokaryotes. Jacob and Monod (1961) proposed the operon theory based on their work with lactose metabolism in Escherichia coli. Briefly, this system contains a regulatory gene which produces a repressor protein that, in the absence of an inducer, binds to a operator gene. This binding prohibits the transcription of structural genes required for metabolism (Jacob and Monod, 1961). Although identical models have not yet been identified in eukaryotes, evidence suggests that analagous systems utilizing specific proteins as regulator molecules are possible (Norman and Bekhor, 1981; LeParco et al., 1981; Perle et al., 1982).

This study was designed to investigate changes in NHC proteins during differentiation in a model system, the 3T3-L1 preadipocyte cell line. These cells were cloned from murine 3T3 fibroblasts (Green and Kehinde, 1974) and are characterized by their ability to differentiate in monolayer culture into cells that exhibit the morphological and biochemical characteristics of adipocytes (Green and Kehinde, 1974, 1975, 1975; differentiation is marked by dramatic changes in the enzyme and protein complement of the cell. Sidhu (1979) has demonstrated that more than 60 new cellular proteins and possibly several new NHC

proteins are synthesized during the differentiation process. An observed change in specific NHC proteins during differentiation would be consistant with a role in gene regulation.

LITERATURE REVIEW

When considering mechanisms of gene expression in eukaryotic cells it is necessary to understand the structure and functional relationships between components of eukaryotic chromatin. Early models of chromatin organization were based on the realization that long uninterrupted stretches of DNA would pose problems for packaging genetic material in the nucleus especially during the condensed conditions of mitosis and DNA replication. This resulted in the idea that each chromosome consisted of many duplex molecules of DNA that were linked end to end by proteins (Freese, 1958). The proteins were supposedly involved in condensing the chromatin and breaks along the duplex of each molecule resulted in the unwinding and replication of the DNA. Another model (Mirsky and Ris, 1951) proposed that chromosomes consisted of a longitudinal backbone of protein to which DNA molecules were attached. With the light microscope, observations of chromosomes prior to mitotic condensation revealed the chromatin to be composed of a single coiled thread. This thread was then examined with the electron microscope and seen to consist of twisted fibers in the extended condition of meiotic prophase (Ris and Chandler, 1963). Early models viewed the fibers as 50-60nm thick made up of two smaller fibers of about 20um in diameter (Lewin, 1980). Later observations indicated that the chromosome is composed of a single fiber of about 30nm diameter. It is now believed that this fiber contains only a single duplex molecule of DNA. Support for this idea is provided by several studies, an early study by DuPraw (1965) showed that treatment with trypsin reduced fibers

to a single filament exhibiting the dimensions of the DNA duplex; treatment with DNAse degreded these filaments. Later studies revealed the 30nm fiber to be a higher level of chromatin organization. Further work with eukaryotic nuclei utilizing electron microscopy showed that most chromatin has a repeating structure of beads about 10nm in diameter connected by a string of DNA (Olins and Olins, 1974). It was later realized that this "beads on string" appearance was not the real structure of chromatin but resulted from the method of sample preparation which removed histone H1. With sample preparation procedures that did not remove H1, the beads were seen not to be stretched out but to form a 10nm fiber with the beads touching each other (DeRobertis and DeRobertis, 1980). These beads became known as nucleosomes and represent the first level of chromatin organization (Lewin, 1980).

The existence and organization of the nucleosome was further elucidated by several studies. Chromatin digestion studies utilizing nucleases demonstrated that DNA was cut into units which were discovered to be lengths of DNA base pairs in multiples of 200 (Noll, 1974). Other workers reported that Histones H3 and H4 associated in solution to form tetramers consisting of two of each histone H3 and H4 (Kornberg and Thomas, 1974). These studies led Kornberg (1974) to propose a nucleosome model in which four histones (H2A, H2B, H3 and H4) form an octamer containing two of each of these histones. Octamers occur every 200 base pairs of DNA with the DNA coiling around the octamer.

The so-called "one-two-three" experiment of Finch et al. (1976) confirmed the nucleosome subunit to be the bead structure first seen with the electron microscope. In this experiment, chromatin was

partially digested with micrococcal nuclease to yield monomer, dimer, trimer, and tetramer fractions which were isolated on a sucrose gradient. Electrophoretic analysis revealed these fractions to contain DNA fragments 200, 400, 600, and 800 base pairs long. Electron microscopy observations of the same fragments revealed them to be composed of one, two, three, and four bed structures respectively. It was realized that micrococcal nuclease cuts DNA into discrete fragments because the linker regions between nucleosomes are exposed to the enzyme. It was also found that extensive enzyme digestion leads to degredation of the linker region leaving the nucleosome core particle intact.

Analysis of the core particle by Noll and Kornberg (1977) showed it to contain 140 base pairs of DNA in addition to the histones H2A, H2B, H3 and H4. The absence of histone H1 in this core particle led them to conclude that H1 is normally associated with the linker region. Further studies utilizing x-ray diffraction and electron microscopy have shown the nucleosome to be a flat disk about 11nm in diameter and 5-7um in height where the DNA is found to turn twice around each histone octamer (Finch et al., 1977).

Comparisons made between nucleosomes obtained from different tissues have shown that the length of DNA per nucleosome may vary (Morris, 1976). This variability has been shown to occur in the linker region as all nucleosome cores contain the same number of DNA base pairs (Cartwright et al., 1982).

The fact that nucleosomes associate to form the 30nm fiber described above has been confirmed (Ris and Kornberg, 1979). How the nucleosomes are organized relative to one another to form this thick fiber has been the subject of much experimentation and debate resulting in two

schools of thought. One hypothesis, originating from the electron microscopic observations of Finch and Klug (1976), proposes that nucleosomes associate to form a regular solenoid of fixed dimensions. It is believed that each turn of the solenoid contains 6 to 7 nucleosomes resulting in a fiber with a 25 to 30nm diameter (Finch and Klug, 1976). The alternative hypothesis proposes that clusters of nucleosomes (from 7 to 20) associate to form a supranucleosomal package called the "super bead" (Hoazier et al., 1977). The super bead is thought to be stabilized as a unit by histone H1 and non-histone proteins. Considerable amounts of evidence favor the former hypothesis (Sperling and Klug, 1977; Suau et al., 1979; Thomas et al., 1980; Lee et al., 1981). This model enables the DNA to exist in a state that is about 40 fold more compact than if stretched out (Finch and Klug, 1976). The DNA found in the metaphase chromosome is reported to be packed between 5,000 and 10,000 times (Finch and Klug, 1976). This suggests that the 30nm fiber must be at least 100 times more folded; the mechanisms by which this is accomplished have not been elucidated at this time.

In interphase nuclei and metaphase chromosomes the 30nm fiber is reported to be folded into loops termed domains (Igo-Kemenes et al., 1972). Sections of fibers composed of 35-85 kilobase pairs of DNA are believed to be looped and anchored to the nuclear membrane or, in the metaphase chromosome, to a protein scaffold (Adolph et al., 1977). Evidence for the existence of domains has been obtained from centrifugation (Nakane et al., 1978), electron microscopy (Lepault et al., 1980), and nuclease digestion studies (Igo-Kemenes and Zachau, 1978). The domain model represents a high order of chromatin structure that presents several possibilities. Domains are thought by some

to be units of replication and transcription (Stalder et al., 1980; Diaz et al., 1981; Stephenson et al., 1981; Cartwright et al., 1982). If they are functional units, their structure and activation may play important roles in developmental regulation (Igo-Kemenes et al., 1982).

HISTONES

As described above, histones are chromatin proteins that help form the nucleosome core particle and linker region. Histones are small (11,000-20,000 m.w.) basic proteins that contain ten to twenty percent arginine and lysine (DeRobertis and DeRobertis, 1980). The four main histones (H2A, H2B, H3 and H4) are structurally very similar in a variety of different species (DeLange and Smith, 1971). For example, the primary structure of rat histone H3 differs only in four amino acids from H3 found in peas (Patthy et al., 1973). Histone H1 displays the most variability of all the histones (Kinkade and Cole, 1966; Bustin and Cole, 1969; Rall and Cole, 1971). The molecular weight as well as the amino acid sequence varies from tissue to tissue in a given species and, with respect to the same tissue, variability occurs from one species to another (Isenberg, 1979). However, H1 contains certain areas that are highly conserved and it is regarded by some to be an evolutionary hybrid (Isenberg, 1979).

Histones are known to undergo postsynthetic modification in several ways. Recent investigations have revealed that acetylation, phosphorylation, methylation and Poly ADP ribosylation occur to histones within the nucleus (Isenberg, 1979; Lewin, 1980). The acetylation and methylation of histones H2A, H2B, H3 and H4 is generally believed to occur during S phase of the cell cycle, suggesting that these modifications

are involved only in chromatin replication (Lewin, 1980). The phosphorylation of histones occurs during S phase, mitosis, and as a response to hormonal stimulation (Isenberg, 1979). Phosphorylation has been reported to be generally of two types: cAMP dependent phosphorylation and growth dependent phosphorylation (Isenberg, 1979). Several investigators have studied phosphorylation during mitosis in synchronized cultures of a variety of mammalian cell lines (Lake and Salzman, 1972; Lake, 1973; Marks et al., 1973; Balhorn et al., 1975). In these cells, H1, H2A, and H3 are all phosphorylated. The phosphorylation of H2A remains constant throughout the cell cycle but phosphorylation of H2 and H3 varies with the cycle stage (Balhorn et al., 1975). These results led Gurley et al. (1974) to propose that different levels of chromatin organization might be associated with different sites of phosphorylation. Histone H1 phosphorylation could be related to changes in organization at the molecular level, H2A phosphorylation could cause heterochromatin condensation and H3 phosphorylation could lead to the condensation of chromosomes. This idea stems from the correlation of phosphorylation with cell cycle variations but no cause and effect relationships have been established (Isenberg, 1979). Nishizuka et al. (1967) suggested that poly ADP-ribose could be covalently linked to histones H1, H2A, H2B, and H3 of rat liver. Poly ADP-ribosylation has been reported to occur to a number of mammalian histones (Smith and Stocken, 1974; Ueda et al., 1975) as well as sea urchin (Ord and Stocken, 1977) and trout testis histones (Wong et al., 1977). It has been suggested that ADP-ribosylation modifies the interaction of histones thereby altering the arrangement of chromatin within the nucleus (Isenberg, 1979).

It was once thought that histones were specific gene repressors (Stedman, 1950). It is obvious that histones are postsynthetically modified in several ways but the fact that histones are highly conserved evolutionarily and lack species or tissue specificity suggests that they are structural elements common to all eukaryotes (Lewin, 1980). Modification of histones are therefore probably mechanisms by which structure is adjusted to the changing demands of housekeeping within chromatin.

NON-HISTONE CHROMATIN PROTEINS

Early investigations using gel electrophoresis techniques were able to detect about 20 different NHC proteins in eukaryotic chromatin (Lewin, 1980). Similar results were obtained in many comparisons of NHC proteins from different sources. These sources included different mammalian tissues, growing and non-growing cells, virally transformed and non-transformed cells, and cells at different stages of development (Teng, 1971; Elgin and Hood, 1973; Platz, 1973; Sanders, 1974). In each case, comparisons of electrophoresis patterns demonstrated that the complement of proteins remained constant among cell types indicating that NHC proteins were common structural components of all cells (Lewin, 1980). However, the advent of more sensitive methods of chromatin analysis has led researchers to conclude that NHC proteins function in several ways. The NHC proteins are thought to include enzymes required for DNA replication, enzymes involved in the histone modifications described above, proteins contributing to the structural integrity of the chromosome, and proteins involved in gene regulation (Bradbury, 1982).

The increased resolution offered by one-dimensional sodium dodecyl sulfate (SDS)-tris-glycine gel electrophoresis has allowed the identification of as many of 100 bands (Lewin, 1980) and in two dimensional gel electrophoresis as many as 450 spots of non-histone chromatin proteins have been counted (Peterson and McConkey, 1976). Structural proteins are prominant non-histone proteins accounting for as much as 40% of the total mass in some cells; among these are actin, myosin, tubulin, and tropomyosin (Douvas, 1975; Bonner, 1975). The most extensively studied group of NHC proteins at present are the high mobility group (HMG) proteins.

The HMG proteins were named by Goodwin et al. (1973) because they exhibited a high mobility relative to other NHC proteins during polyacrylamide gel electrophoresis, resulting in their accumulation near the bottom of the gel. These proteins were first extracted from calf thymus chromatin and were further characterized by Johns (1982) as "chromatin proteins, extractable from chromatin in 0.35M NaCl, not precipitated from 0.35M NaCl by 2% trichloroacetic acid, high in basic amino acids like the histones, high in acidic amino acids unlike histones, and relatively high in proline". Four mammalian HMG proteins have been identified thus far and are named HMG 1, 2, 14 and 17. The HMG 1 and 2 have molecular weights of about 27,500 and 26,000 daltons respectively (Goodwin et al., 1973) and have similar amino acid sequences and structural properties (Bradbury, 1982). The HMG 14 and 17 have molecular weights of around 14,000 and 9,000 daltons respectively and also share similar structures (Johns, 1982).

Several studies have suggested that HMG 1 and HMG 2 are associated with actively transcribing chromatin (Jackson et al., 1981; Georgieva

et al., 1981). However, this idea is contradictory to reports that high levels of HMG 1 and 2 exist in transcriptionally less active cells (Goodwin et al., 1979). Seyeclin et al. (1981) suggest a correlation exists between the levels of HMG 1 and 2 and the stages of differentiation of Friend's erythroleukemia and mouse neurobastoma cells. It is reported that levels of HMG 1 and 2 are decreased in these cells when they are induced to differentiate. The HMG 1 and 2 have also been shown to preferentially bind to single stranded DNA (Bidney et al., 1978). Javaherian et al. (1979) report that HMG 1 and 2 cause a net unwinding of supercoiled DNA. Considering current data, Goodwin and Mathew (1982) have suggested that a possible role for both HMG 1 and 2 is to aid in the unwinding of DNA for replication and transcription.

The HMG 14 and 17 seem to be functionally unrelated to HMG 1 and 2 (Goodwin and Mathew, 1982). It is now believed that HMG 14 and 17 bind to core nucleosomes and effect nucleosome structure (Cartwright et al., 1982). Digestion of chromatin to mononucleosomes containing either 145 or 160 base pairs of DNA indicated that the 160 base pair section retained both HMG 14 and 17 while the 145 base pair segment retained neither (Goodwin et al., 1979). Mardian et al. (1980) demonstrated that nucleosome cores have two specific binding sites for HMG 14 and 17. Weisbrod and Weintraub (1979) presented evidence for the possible involvement of HMG 14 and 17 in chromatin transcription, demonstrating that chromatin from embryonic chicken erythrocytes extracted with 0.35M NaCl displayed no globin gene sensitivity to DNAse I digestion. Upon reconstitution of chromatin with the 0.35M NaCl extract or with HMG 14 and 17, full DNAse I sensitivity was restored to the

globin gene. Considering all present data, Goodwin and Mathew (1982) suggest that HMG 14 and 17 may function in a mechanism that *in vivo* leads to a more open DNA structure thereby increasing the progress of RNA polymerase during transcription. The HMG proteins probably play important roles in chromatin organization and function as evidenced by the studies cited above but are probably not direct regulators of gene expression in eukaryotes. This leaves the remaining NHC proteins for consideration as gene regulators.

In a number of cell systems, changes in the complement of NHC proteins has been observed during the differentiation and growth process (Keppel *et al.*, 1977; Rodriguez *et al.*, 1979; Perle and Neuman, 1980; Heizman *et al.*, 1980; Morioka *et al.*, 1981; Adolph and Phelps, 1982; Perle *et al.*, 1982). Morioka *et al.* (1981) reported that during differentiation of induced friend erythroleukemia cells, chromatin NHC protein contents decreased 50% upon induction of differentiation. Keppel *et al.* (1977) demonstrated that induction of differentiation in Friend cells is accompanied by the appearance of a new chromosomal protein with an apparent molecular weight of 25,000 daltons. This protein [which Keppel *et al.* (1977) named Ip_{25}] was found to be a major constituent of chromatin and appears tightly bound to DNA. The analysis of NHC proteins during cell dedifferentiation (giving rise to regeneration cells) during traumatic regeneration in the polychaete annelid *Owenia fusiformis* indicates that several changes occur in the population of NHC proteins (LeParco *et al.*, 1981). Genetic deprogramming which occurs during dedifferentiation is reported to be accompanied by an overall increase in the number of NHC proteins as well as an increase in phosphorylation of these proteins (LeParco *et al.*, 1981). Perle

and Neuman (1980) reported that two NHC proteins with molecular weights of 125,000 and 35,500 daltons disappear from chromatin of embryonic chicken precartilage mesenchyme cells as they differentiate into cartilage. Further studies by Perle and Neuman (1982) on abberant embryonic chicken precartilage cells revealed that two NHC proteins with molecular weights of 120,000 and 36,500 daltons replaced the 125,000 and 35,500 dalton proteins observed in normal precartilage cells. The analysis of chromosomal proteins from rat hepatocytes and hepatocellular carcinomas revealed that as many as 10 NHC proteins ranging in molecular weight from 55,000 to 220,000 were present in carcinomas but absent in normal cells (Rodriguez et al., 1979). It was also reported that rapidly growing tumors possessed more NHC protein than did slow growing tumors (Rodriguez, et al., 1979). In vivo studies of the differentiation of cerebellar neurons revealed that two NHC proteins with molecular weights of 35,000 and 38,000 appear at the approximate time of the cells transition from a proliferative to a non-proliferative state (Heizman et al., 1980). Further developmental alterations occurring during the same period were the disappearance of a major protein with a molecular weight of 15,000 daltons and the increased expression of a group of proteins with molecular weights of 40,000 to 70,000 daltons (Heizman et al., 1980).

Other studies utilizing different mthods than the direct comparison of protein complements during new gene expression lend further support to the hypothesis that NHC proteins are involved in gene regulation. Kamiyama and Wang (1971) demonstrated that NHC proteins partially purified from rat liver chromatin stimulate chromatin-templated RNA synthesis in vitro. They report that RNA transcribed from chromatin

stimulated by the NHC proteins has a different nucleotide composition than RNA transcribed from control chromatin. Similar studies using different *in vitro* transcription models show similar results (Teng and Hamilton, 1969; Wang, 1970; Chiu *et al.*, 1975). Norman and Bekhor (1981) isolated chromatin from the human placenta and after extraction with 2M NaCl found that the DNA separated into two components: a protein free component consisting of 96% of the total DNA and a protein rich component consisting of 4% of the total DNA complexed with tightly bound NHC proteins. Reassociation studies revealed that the latter fraction was enriched 22 fold in actively transcribed human placental lactogen gene sequences. Further analysis revealed that 27% of this DNA was DNAse I sensitive lending further support that the DNA contained actively transcribed gene sequences (Norman and Bekhor, 1981).

Much evidence has been presented in the literature that supports the hypothesis that NHC proteins are involved in gene regulation. The purpose of this study was to look at changes in the complement of NHC proteins during differentiation in a model system, the 3T3-L1 preadipocyte, as a first step towards understanding the role of NHC proteins in gene regulation during differentiation.

THE 3T3-L1 PREADIPOCYTE

The 3T3-L1 preadipocyte was derived from the 3T3 murine fibroblast line (Todaro and Green, 1963). The 3T3 fibroblast line was first isolated from mouse embryo and is characterized by its ability to differentiate into cells which accumulate lipid (Green and Kehinde, 1974; Green and Meuth, 1974). By starting with clones from the 3T3 cell line, Green and Kehinde (1974) were able to generate subclones which

displayed a high frequency of conversion to the fatty phenotype. Twenty clones were isolated from 3T3 stock, 19 of which were found to have the ability to develop into adipose-like cells. One subclone that displayed an extraordinary tendency to accumulate lipid was labeled the 3T3-L1 preadipocyte (Green and Kehinde, 1974). Another subclone labeled 3T3-C2 was isolated and characterized by its inability to differentiate to the fatty phenotype (Green and Kehinde, 1974). Under normal conditions, the 3T3-L1 preadipocyte will convert to adipocyte cells after 3 to 4 weeks. However, Rubin et al. (1978) reported that treatment with insulin, dexamethasone, and 1-methyl-3-isobutylxanthine induces differentiation.

The transition from preadipocyte to adipocyte cells is marked by the synthesis and deposition of cytoplasmic fat. During adipocyte conversion, 3T3-L1 cells exhibit a coordinate rise in almost all the enzymes of de novo fatty acid synthesis (Mackall et al., 1976) and triacylglycerol synthesis (Coleman et al., 1978). Moreover, it has been demonstrated by Sidhu (1979) that the synthesis of more than 60 cellular proteins is greatly enhanced during this differentiation process. Conversion to the fatty phenotype is also accompanied by striking morphological changes. The once elongated, stellate shaped cells retract their cellular processes and take on a spherical shape containing numerous lipid droplets when differentiation occurs (Green and Kehinde, 1976).

Several characteristics of the 3T3-L1 preadipocyte presented thus far make this an ideal model system for looking at molecular events occurring during differentiation:

1. The 3T3-L1 preadipocyte can be grown on monolayer culture and induced to differentiate by the addition of several agents.
2. Differentiation is marked by dramatic changes in both morphological and biochemical characteristics which are easily observed in the laboratory.
3. Because the 3T3-C2 cell line does not exhibit any degree of differentiation when exposed to conditions that induce differentiation in 3T3-L1 preadipocytes, this can be used as a control line.
4. Since the expression of the adipocyte results in the synthesis of specific new proteins, it is expected that alterations in chromatin structure and in transcription are early events in the process.

MATERIALS AND METHODS

MATERIALS

Dulbecco's modified Eagle's medium was obtained from GIBCO Laboratories (Grand Island, NY). Fetal bovine and calf serum were purchased from Sterile Systems (Logan, UT), methyl-isobutyl-xanthine (MIX) was obtained from Aldrich (Milwaukee, WI), dexamethasone (DEX) was from Sigma Biochemicals (St. Louis, MO), and insulin was obtained from Eli Lilly (Indianapolis, IN). Phenylmethylsulfonyl floride (PMSF) was purchased from Calbiochem (San Diego, CA), [^{35}S] methionine was purchased from Amersham (Chicago, IL), [^{3}H] leucine was purchased from ICN Nutritional Biochemicals (Cleveland, OH) and En^{3}Hance was obtained from New England Nuclear (Boston, MA). Ampholines for electrophoresis were purchased from Bio Rad (Richmond, CA) and all cell culture plates and disposable plastics were purchased from Falcon (Oxnard, CA). The remaining common laboratory reagents were purchased from either Sigma Scientific or Fisher Scientific Company (Pittsburg, PA).

CELL CULTURE

3T3-L1 and 3T3-C2 cell lines were originally obtained from Dr. M. Daniel Lane, Department of Physiological Chemistry, the Johns Hopkins University School of Medicine and were routinely maintained as follows: cells were plated at 1000 cells per cm^2 in Dulbecco's modified Eagle's medium containing 10% calf serum. Cells were cultured in a humidified

atmosphere of 10% CO_2/90% air at 37°C using a New Brunswick CO 21 incubator. Culture medium was replaced every 48 hours. At two days post confluence the cells were placed in fresh medium supplemented with 10% fetal bovine serum and adipose conversion was inititated by the addition to culture of MIX, DEX, and insulin. The induced cell population would then undergo 1-2 rounds of division. Forty-eight hours after addition, the agents were removed and the cells maintained in the presence of medium supplemented only with 10% fetal bovine serum and insulin. Cell growth and acquisition of the adipocyte phenotype was monitored by microscopic examination of the cell monolayer using an Olympus inverted phase contrast microscope. Cell number was determined utilizing a hemacytometer counting chamber.

CELL HARVEST

Both 3T3-L1 and 3T3-C2 cells were harvested from culture at the desired time point by first rinsing each monolayer with ice cold phosphate buffered saline (PBS) which contained 0.9 mM $CaCl_2$, 2mM KCl, 0.5mM $MgCl_2.6H_2O$, 130 mM NaCl, 5.8 mM $Na_2HPO_4.7H_2O$ (pH 7.4). (From this point on, all materials were maintained at 4°C). Then the cells were removed from the plates by scraping with a rubber cell scraper in the presence of 2 ml of PBS; the resulting cell suspension was then transfered to a 15 ml polystyrene centrifuge tube and the cells pelleted by centrifuging in a Dynac clinical centrifuge at a setting of 60 for 4 minutes.

EXTRACTION OF CHROMATIN

Nuclei were isolated by a modification of the method of Keppel et al. (1977) by disrupting cells in a hypotonic solution (0.01M NaCl, 0.01M Tris, 0.0015M $MgCl_2$-$6H_2O$, 1mM PMSF) with a tight fitting Dounce homogenizer (70 strokes) followed by centrifugation at 1000 x g for 7 minutes. The isolated nuclei comprising the pellet were washed once in the same buffer followed by centrifugation at 1000 x g for 7 minutes. The washed nuclei were then lysed by gentle homogenization in 10mM Tris-HCl (pH 8), 25mM ethylene-diaminetetraacetate (EDTA), 0.5% Triton X-100, and 1mM PMSF followed by centrifugation at 1000 x g for 7 minutes. The pellet was then washed with 5mM Tris-HCl (pH 8). Following centrifugation at 1000 x g for 7 minutes, the pellet was resuspended in 2ml of 5mM Tris-HCl (pH 8) and centrifuged through a solution of 65% sucrose in 5mM Tris-1mM PMSF for 70 minutes at 80,000 x g. The resulting chromatin pellet was washed once with 10mM Tris-HCl (pH 7.4) containing 1mM PMSF and 1mM EDTA. The chromatin pellet was then either used immediately or stored at -80°C. During this procedure, low speed centrifugation was performed on an International Centra-7R while high speed centrifugation was carried out on a Beckman model L5-75 Ultracentrifuge.

EXTRACTION OF CHROMATIN PROTEINS

Chromatin proteins were isolated from the purified chromatin by the method of Chiu et al. (1977). A chromatin pellet was gently homogenized in 3ml of 5.0M urea, 2.5M NaCl, 50mM Tris-HCl (pH 8.0) followed by stirring for 2-3 hours. The resulting mixture was then centrifuged at 110,000 x g for 72 hours. The supernatant containing proteins was

then removed and the pellet composed of DNA discarded.

PREPARATION OF PROTEINS FOR SDS-POLYACRYLAMIDE GELE ELECTROPHORESIS

The 3ml supernatant containing the extracted chromatin proteins was dialyzed for 24 hours against 3 liters of 2mM Tris (pH 7.5), 0.2mM PMSF. The buffer was changed once after approximately 12 hours. Dialysis was carried out using Spectropore membrane tubing with a molecular weight pore size of 6,000 to 8,000. It should be noted that most of the chromatin proteins precipitated during this procedure. Following dialysis, the sample was transfered to a 12 ml conical glass centrifuge tube and frozen by immersing in a mixture of dry ice and methanol. The sample was immediately freeze-dried using a VirTis lyophilizer. The resulting protein sample was dissolved in 150 µl of electrophoresis sample buffer containing 20mM Tris-HCl, 0.5% SDS, 10% glycerol, and 2% betamercaptoethanol (BME). Protein concentration was determined for each sample using a turbidity protein assay as described by Dr. S. Hnilica (personal communication). A 10 µl aliquot of resuspended protein was added to 0.5 ml of 0.5M urea followed by the addition of 0.5 ml of 50% trichloroacetic acid (TCA). The absorbance was read at 400 nm on a Guilford model 260 spectrophotometer. These results were compared to a standard curve generated by using various concentrations of bovine serum albumin (BSA) in place of sample in the above procedure. Prior to electrophoresis, samples were placed in a boiling water bath for 3 minutes.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Discontinuous SDS-polyacrylamide gel electrophoresis was carried out using slab gels with 120mm x 106 mm x 1 mm dimensions; these gels were made using the standard Lammeli (1970) procedure. Separating gels consisting of filtered 12% acrylamide, 0.1% SDS, and 0.375 M Tris-HCl (pH 8.8) were mixed and polymerization initiated by the addition of 0.003 volumes of 10% ammonium persulfate and 0.001 volumes of N, N, N', N'-tetra-methylethylenediamine (TEMED). This solution was immediately poured into the gel plate mold to a height of 100 mm and overlayed with water saturated isobutanol. After one hour, the water saturated butanol was removed and replaced with gel buffer (0.37 M glycine, 0.074 M Tris-HCl (pH 6.8), 1% SDS). After polymerization was complete, gel buffer was removed from the gel, a sample well forming comb was inserted, and stacking gel (4.75% acrylamide, 0.1% SDS, 0.125 M Tris-Cl, pH 6.8) was mixed and poured as above. The stacking gel was allowed to polymerize for 30 to 60 minutes at which time the sample well comb was removed and the gel was attached to the electrophoresis cell. Samples containing equal concentrations of protein as determined by the turbidity assay described previously were applied to the sample wells. Electrophoresis was carried out at 15-25 ma through the resolving or separating gel. All gels included at least one lane utilized for standards of known molecular weight and 0.001 bromphenol blue added as a tracking dye. Gels were run for approximately 5 hours or until the tracking dye reached approximately 1 cm from the gel bottom. Electrophoresis was conducted using a Bio Rad model 220 electrophoresis cell and a Heathkit model SP-2717 power supply.

STAINING OF POLYACRYLAMIDE GELS

After electrophoresis, gels were stained using the modified procedure of Merril et al. (1981). Gels were fixed in 400 ml of 40% methanol, 10% acetic acid for 30 minutes to overnight. This was followed by 2 washes (15 minutes duration each wash) of 10% ethanol, 5% acetic acid. Gels were then soaked for 5 minutes in a 200 ml solution of 0.0034 M potassium dichromate and 0.0032 N nitric acid, washed twice for 5 minutes in deionized water, and place in 300 ml of 0.012 M silver nitrate for 20 minutes under a florescent light source. The gels were then rinsed once rapidly with 250 ml deionized water followed by 250 ml of image developer solution containing 0.28 M sodium carbonate and 0.5 ml formaline per liter. This solution was changed after 30 seconds and the image allowed to develop. When the image reached the desired intensity, development was stopped by discarding the developer and adding 400 ml of 5% acetic acid for 5 minutes. Gels were agitated gently throughout the procedure using a Fisher shaking water bath.

RADIOLABELING OF CYTOSOLIC PROTEINS WITH [^{35}S] METHIONINE

The medium was aspirated off one 6 cm plate of fully differentiated 3T3-L1 cells. The cells were then washed twice with pre-equilibrated methionine deficient media followed by the addition of 2 ml of media containing 330 μCi [^{35}S] methionine. The cells were pulsed for one hour in the incubator after which the labeled cytosolic proteins were extracted according to the procedure of Mackall et al. (1980). This was accomplished by first washing the cells twice with 3 ml of ice cold PBS followed by the addition of 2.0 ml of buffered digitonin (17

mM morpholinopropanesulfonic acid, 2.5 mmM EDTA, 0.25 M mannitol, 0.65 mM digitonin) which was allowed to sit for 10 minutes at which time it was aspirated off gently. A 20µl sample was then taken from the release to determine the amount of label incorporation into protein. The sample was added to 0.5 ml of ice cold 20% TCA with 25µl of 0.5% BSA added as a carrier. After setting at 4° C for 1 hour, the mixture was centrifuged at 2,000 x g for 5 minutes in an International centrifuge. The pellet obtained was solubilized by incubation in 0.5 ml of 1M NH_4OH at 37°C for 30 minutes. The protein was then reprecipitated on addition of 5.0 ml of ice cold 10% TCA and filtered on Whatman GF/C filters. The filter was then extracted with diethyl ether and the radioactivity present determined using a Beckman scintillation counter.

Thirteen 6 cm plates of differentiated cells were then harvested and pelleted. Labeled cytosolic proteins were added to these cells and the chromatin proteins extracted as previously described. These proteins were then run on 15% polyacrylamide gels as described above. Labeled protein standards were applied to one gel lane. Following electrophoresis, gels were fixed and stained overnight in 25% isopropanol, 10% acetic acid, and 0.05% Coomassie brilliant blue. This was followed by destaining in 10% isopropanol, 10% acetic acid. The gel was then treated with En^{3}Hance autoradiography enhancer, dried, and exposed to Kodak X-omat R film for thirty days.

RADIOLABELING OF CHROMATIN PROTEINS WITH [4,5^{3}H] LEUCINE

Twenty plates of confluent 3T3-L1 cells and 15 plates of 3T3-L1 cells were incubated with medium containing [4,5^{3}H] Leucine. Following

the labeling period of 24 hours, the medium was removed, monolayers were washed with ice cold PBS and chromatin proteins were extracted as previously described then separated using polyacrylamide gradient gel electrophoresis.

POLYACRYLAMIDE GRADIENT GEL ELECTROPHORESIS

140 mm x 106 mm 6-15% SDS-polyacrylamide gradient gels were formed using a Pharmacia GM-1 gradient maker. Both 6% and 15% separating gel solutions were made up individually as previously described then added to separate compartments in the gradient maker. The gels were then poured from the gradient maker to a height of 140 mm and overlayed with water soluable butanol. After polymerization, the water soluable butanol was removed and a stacking gel formed as previously described. [4,5^{3}H] leucine labeled samples were then applied to the sample wells (labeled protein standards were applied to one gel lane) and electrophoresis carried out as previously described. Following electrophoresis, gels were either stained first or treated directly with 1M salicylate (an autoradiography enhancer), dried, and exposed to Kodak Xomat-R film for various time periods.

RESULTS

SELECTION OF A METHOD FOR EXTRACTION OF CHROMATIN PROTEINS

Selecting a method to obtain a contamination free sample of adequate size from a reasonable number of cells proved difficult. Several methods of extracting chromatin proteins were tried before a suitable one was found. In preliminary work, chromatin was subjected to DNase digestion (a nuclease which digests DNA leaving free protein) as a method to extract chromatin proteins. This procedure was successful in releasing sufficient protein but the concentration of DNase was so high that upon electrophoresis the DNase protein band obscured many chromatin proteins. In another method chromatin proteins were isolated by a series of extractions with increasing salt concentrations. This required a large number of cells and provided unreliable results. The method of Chiu *et al.* (1977) as described in Materials and Methods was finally selected. This method provided uniformly reproducable results but presented two problems. Because the procedure produced a sample that was dilute, large numbers of cells ($\sim 10^7$) were necessary to obtain a protein concentration suitable for adequate detection of protein bands following electrophoresis. Also the high salt concentration used to extract the proteins resulted in the poor resolution of protein bands when subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. These two problems were overcome by utilizing the method of Schmidt and Hnilca (Personal Communication, W. Schmidt) in which salt was dialysed away and proteins concentrated as described in the Materials and Methods section.

EXAMINATION OF ISOLATED CHROMATIN PROTEIN FOR CONTAMINATION BY CYTOSOLIC PROTEIN

A mixing experiment was performed where 1.6×10^6 counts per minute (c.p.m.) of [^{35}S] methionine labeled 3T3-L1 cytosolic protein was mixed with unlabelled total cellular protein and chromatin protein extracted as described in Materials and Methods. The extracted chromatin protein was subjected to SDS polyacrylamide gel electrophoresis followed by autoradiography (Figure 1). Lanes A1 and B1 contain 9.43×10^4 c.p.m. and 1.88×10^5 c.p.m. respectively of [^{14}C] labelled standards while lanes A2,3 and B2,3 contain 25 μg of the extracted chromatin protein. The autoradiogram was developed after 30 days of exposure and, while lanes containing standards were deliberately over exposed, no radioactive protein was observed in those lanes containing the chromatin extract.

CHARACTERIZATION OF CHROMATIN PROTEINS FROM 3T3-L1 and 3T3-C2 CELLS

Chromatin proteins were extracted from 3T3-C2 cells at three different stages of treatment and analyzed by electrophoresis on 0.1% SDS-12% polyacrylamide gels (Figure 2). Lane 1 represents protein extracted post confluence but prior to addition of 1-methy-3-isobutyl xanthine (MIX), dexamethasone (DEX) and insulin. Lanes 2 and 3 represent time points 12 and 90 hours after treatment with MIX, DEX, and insulin. Approximately 70 distinct bands were stained using the silver stain procedure. The most prominent bands occur in the lower molecular weight range of 20-25,000 daltons (all molecular weights for this study were extrapolated from a graph of the relative mobility of molecular standards [Figure 3]) while another prominent group of protein bands occurs between

35,000 and 40,000 daltons. Other bands appear at 80,000; 68,000; 66,000; 60,000; 53,000; 43,000; 38,000; and 30,000 daltons. When comparing lanes 1, 2, and 3, no distinct differences in banding patterns are noticeable although most bands are more pronounced in lane 2.

In a second experiment, chromatin proteins were extracted from both 3T3-L1 and 3T3-C2 cells at different stages of growth and treatment and subjected to analysis as described above. The results of this study are displayed in Figure 4. Lanes 5 and 6 contain chromatin protein from the 3T3-C2 cells, the banding patterns of which are identical to those shown in Figure 1. Chromatin protein extracted from the 3T3-L1 cells and shown in lanes 1-4 exhibit a marked similarity to those from the 3T3-C2 cells. A major exception is a protein of 30,000 daltons that is first detected at 24 hours after treatment of the cells with MIX, DEX, and insulin and by 90 hours presents a prominent band. This study also demonstrates that 28,000 daltons protein varies in cellular content with respect to time of treatment. This protein band stained very faintly in preconfluent (lane 1) and confluent (lane 2); is absent in cells 12 hours after treatment with MIX, DEX, and insulin (lane 3); is pronounced in cells 90 hours after treatment with MIX, DEX, and insulin; and is absent from 3T3-C2 cells (lanes 5 and 6).

Increased resolution of the higher molecular weight protein bands from both 3T3-L1 and 3T3-C2 cells was obtained by electrophoretic analysis on 0.1% SDS-7% polyacrylamide gels (Figure 5). Prominent bands for both cell types were observed at 50,000; 53,000; 60,000; 66,000; 68,000; 75-85,000; 95,000; 98,000; and above 120,000. In protein derived from the 3T3-L1 cells (lanes 1-4), two proteins of molecular weights 110-120,000

and 150-200,000 daltons are absent from the sample derived from confluent 3T3-L1 cells (Lane 1) but appear as the cells are treated to induce differentiation (lanes 2-4).

For further analysis of low molecular weight chromatin proteins (25-60,000 daltons), extracted proteins from 3T3-L1 cells were analyzed on additional 12% polyacrylamide gels (Figures 6 and 7). Protein bands on these gels were found identical to those seen in other 12% gels of the same dimension, however the amount of one protein of 53,000 daltons was seen to vary with respect to time after treatment with MIX, DEX, and insulin. In Figure 6, this protein becomes prominent 12 hours after treatment (lane 2) and in Figure 7 is more pronounced at 24 and 90 hours after treatment (lanes 3 and 6 respectively). This variability was not seen on any other gel.

Cells were pulse labelled with [3,4-^{3}H] leucine enriched media and harvested prior to treatment with MIX, DEX, and insulin. Additional cells were then pulse labelled similarly and harvested in their differentiated form 90 hours after initial treatment with MIX, DEX, and insulin. Chromatin proteins were then extracted and analyzed on a 0.1% SDS-6% polyacrylamide gradient gel. The resulting gel was first silver stained and analyzed (Figure 10), then cleared of stain, treated with fluor, and exposed to x-ray film. The autoradiogram was then scanned with a densitometer (Figures 8 and 9). The results of the scan are summarized in Table 1. Twenty-five peaks representing chromatin protein were recorded in the scan of proteins from fully differentiated cells (Figure 9). One protein (arrow in Figure 9) of 70,000 daltons was observed in chromatin extracted 90 hours after initiation of differentiation but not seen in

undifferentiated cell chromatin. Although the same amount of radioactivity ($2.5x10^4$ c.p.m.) was applied to both lanes of the gel, it is apparent from comparing Figures 8 and 9 that differences exist between the amount of label incorporated into proteins from undifferentiated compared to differentiated cells. Generally, more label incorporation is seen in the differentiated cell chromatin protein (Figure 9) as many of the peaks are larger than the corresponding ones from undifferentiated cells (Figure 8). However, this is not absolute as several peaks show the opposite trend. Proteins from differentiated cells that show increases in amount compared to undifferentiated chromatin protein include those of peak number (from Figure 8 and 9) and molecular weights: (1) 130,000; (2) 110,000; (7) 75,000; (12) 53,000; (13) 50,000; (14) 48,000; (15) 45,000; (17) 43,000; (18) 41,000; (19) 38,000; and (23) 27,000. Proteins from differentiated cells that show decreases in amount compared to undifferentiated chromatin protein include those of peak number and molecular weight: (3) 100,000; (4) 95,000; (6) 85,000; (8) 73,000; (20) 35,000; and (21) 33,000. It should be noted that the entire histone fraction which contributed the major protein bands on these gels was deleted for this analysis. However, in all experiments performed, no variation in histone content was observed.

Upon examination of the 6-15% gradient gel silver stained prior to autoradiography (Figure 10), it can be seen that several differences exist between undifferentiated and differentiated cell chromatin proteins in the upper molecular weight range. Two bands, one at 200,000 daltons (top arrow) and one at 150,000 daltons (second arrow) appear in lane 2 and not in lane 1. These bands were not distinguishable on the autoradiogram scan of the same gel. The third arrow from the top shows a

band of 100,000 daltons that is seen only in lane 2. One area of "doublet" bands is seen in lane 2 beginning with a band at 100,000 daltons (bottom arrow) and continuing down to include the next two bands. It should be noted that autoradiography and silver stain methods highlighted different bands on the same gel. For this reason, it is difficult to make direct comparisons of Figures 8 and 9 with Figure 10.

To summarize, using gels with several different polyacrylamide concentrations in addition to autoradiography coupled with densitometer scanning, this study has separated a wide molecular weight range of chromatin proteins and shown some major changes to occur in these populations during differentiation. Electrophoresis of these proteins on 12% polyacrylamide gels has shown that one protein of 30,000 daltons which is absent from undifferentiated cells becomes prominent after differentiation. These studies also show proteins with molecular weights of 28,000 and 53,000 daltons which fluctuate in nuclear content over a time course of growth and differentiation. Analysis of higher molecular weight proteins on 7% polyacrylamide gels show two proteins of 110-120,000 and 150-200,000 daltons to arise in chromatin as cells progress toward differentiation. A similar pattern was observed when proteins were subjected to electrophoresis on 6-15% polyacrylamide gradient gels. Bands of 200,000; 150,000; and 110,000 daltons were seen to occur in chromatin from fully differentiated cells but were absent in cells prior to treatment with MIX, DEX, and insulin. Finally, densitometer scanning of a labelled chromatin protein autoradiogram demonstrated variations in the amount of label incorporated protein in differentiated compared to undifferentiated cell chromatin.

Figure 1. Autoradiogram of 3T3-L1 extracted chromatin proteins from [^{35}S] methionine mixing experiment run on 15% polyacrylamide gels (A and B). Lanes: (1) ^{14}C labelled standards; (2) and (3) 25 μg of extracted chromatin proteins.

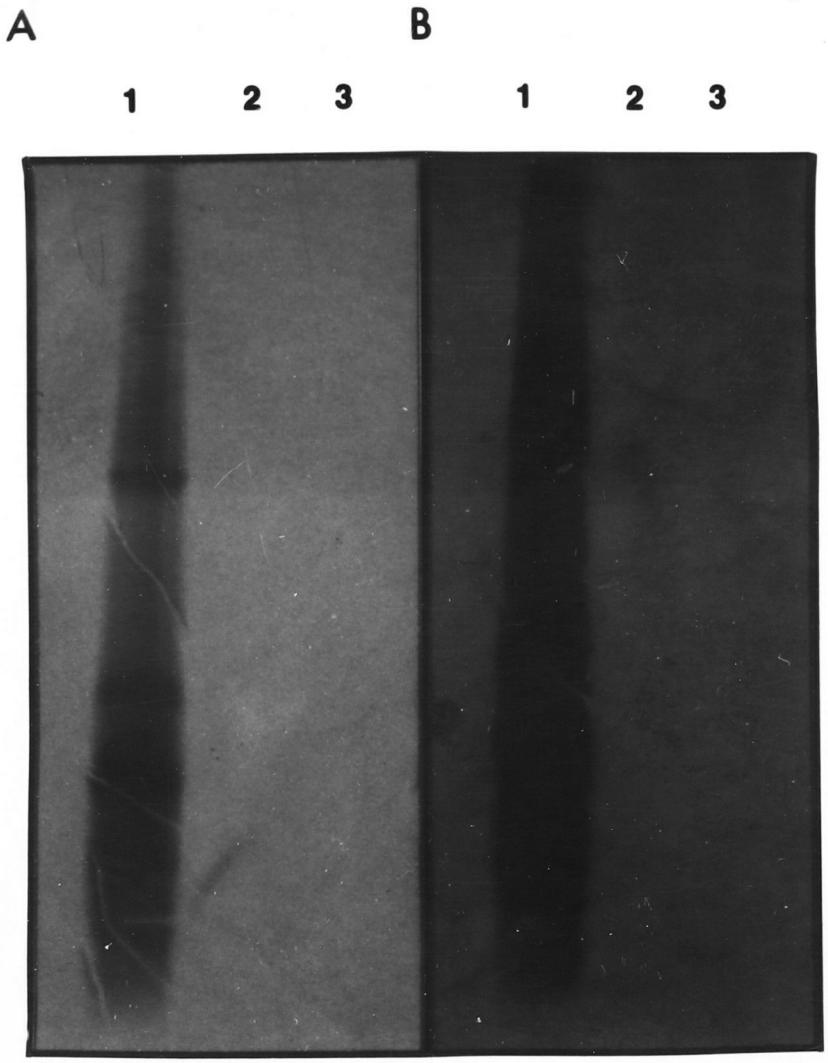
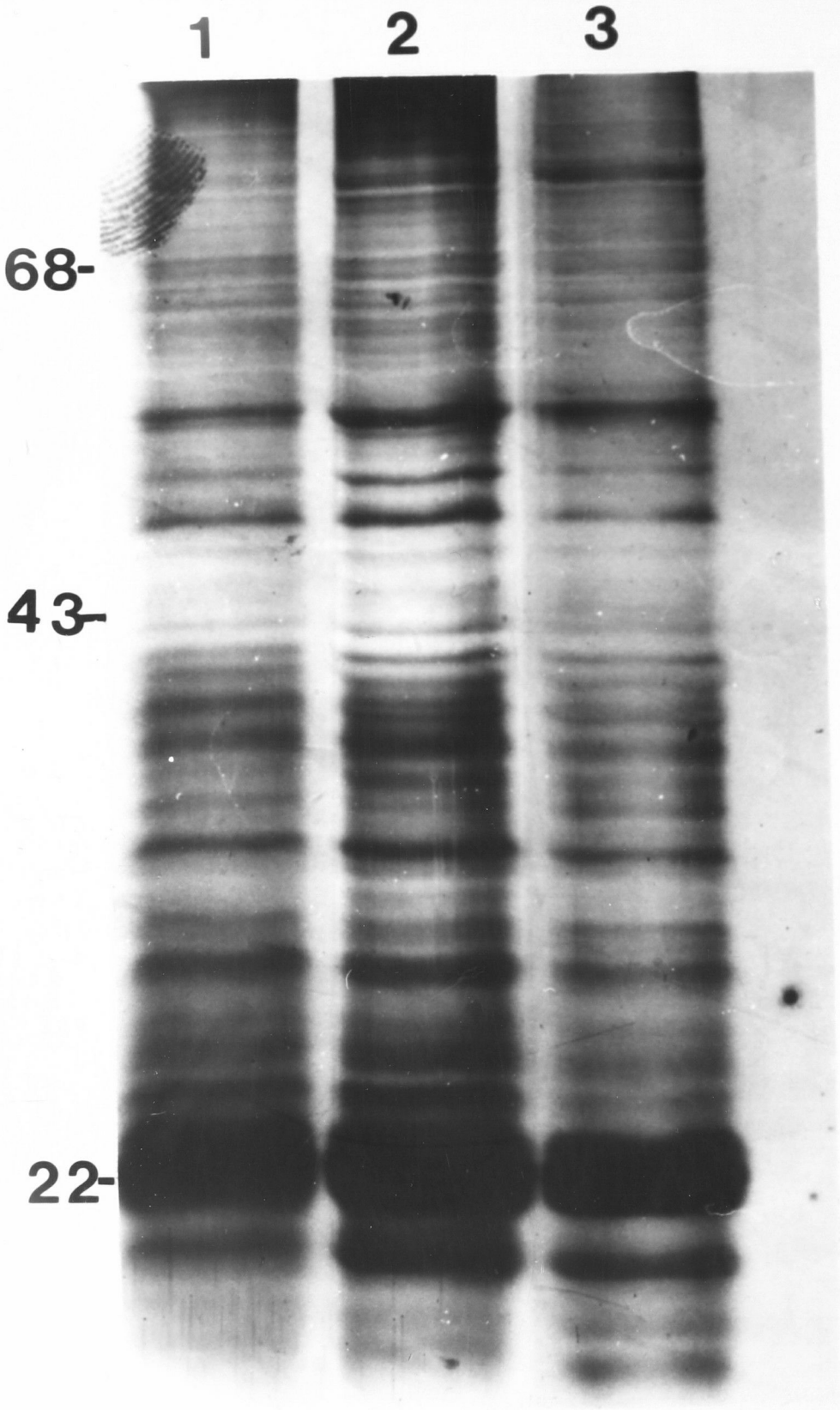

A
B
1
2
3
1
2
3

Figure 2. 0.1% SDS-12% polyacrylamide gel of chromatin proteins extracted from 3T3-C2 cells at three different time points: (1) 5 µg chromatin proteins extracted prior to the addition of MIX, DEX, and insulin; (2) 5 µg chromatin proteins extracted after 12 hours of incubation in the presence of MIX, DEX, and insulin; (3) 5 µg chromatin proteins extracted 90 hours after initial treatment with MIX, DEX, and insulin. Molecular weights of standards ($x10^{-3}$) are shown.


1
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3
68-
43-
22-

Figure 3. Example of graph used to determine molecular weights for this study. Plot of standard molecular weight vs. relative mobility (R_m) on a 12% polyacrylamide gel. Protein standards used in decreasing molecular weight: phosphorylase B, bovine serum albumin, ovalbumin, α-chymotrypsinogen, histone H1.

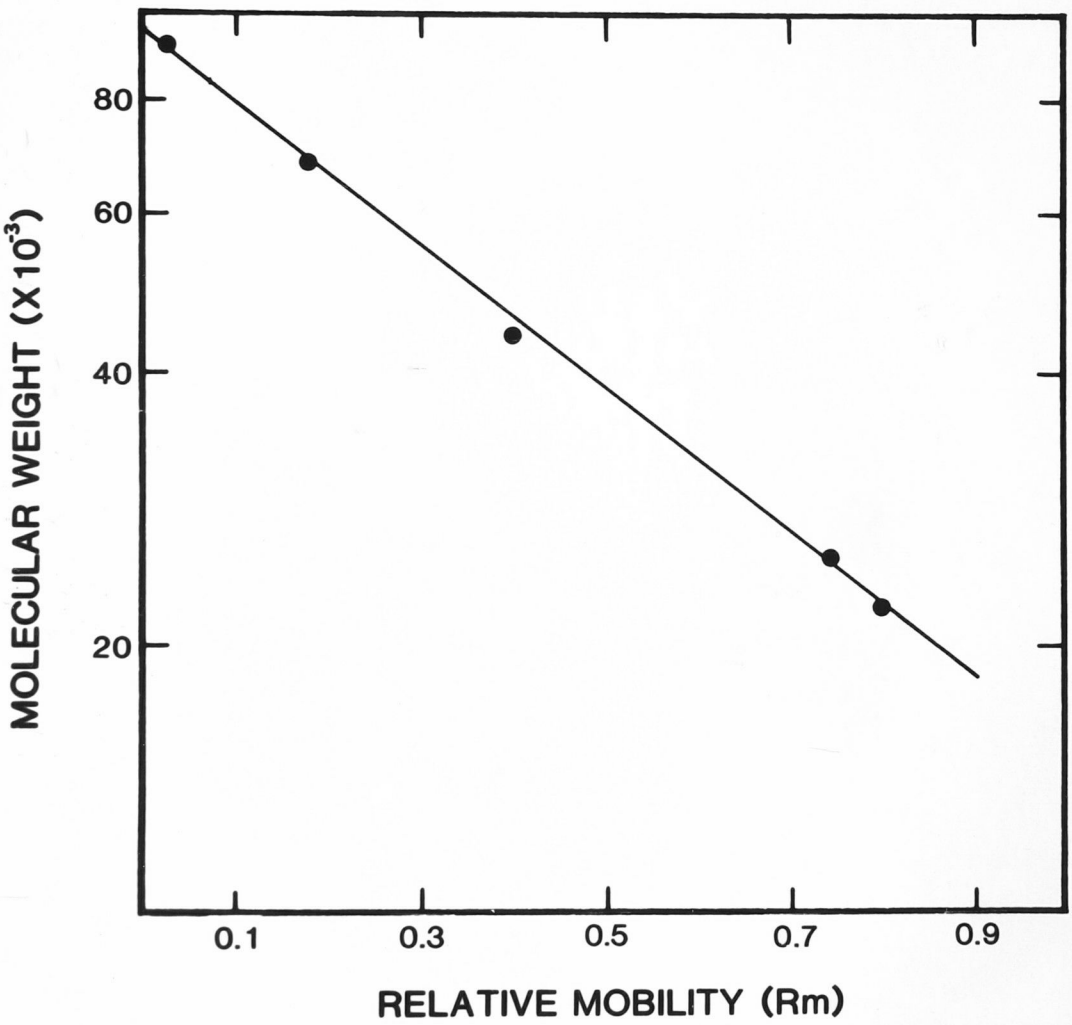
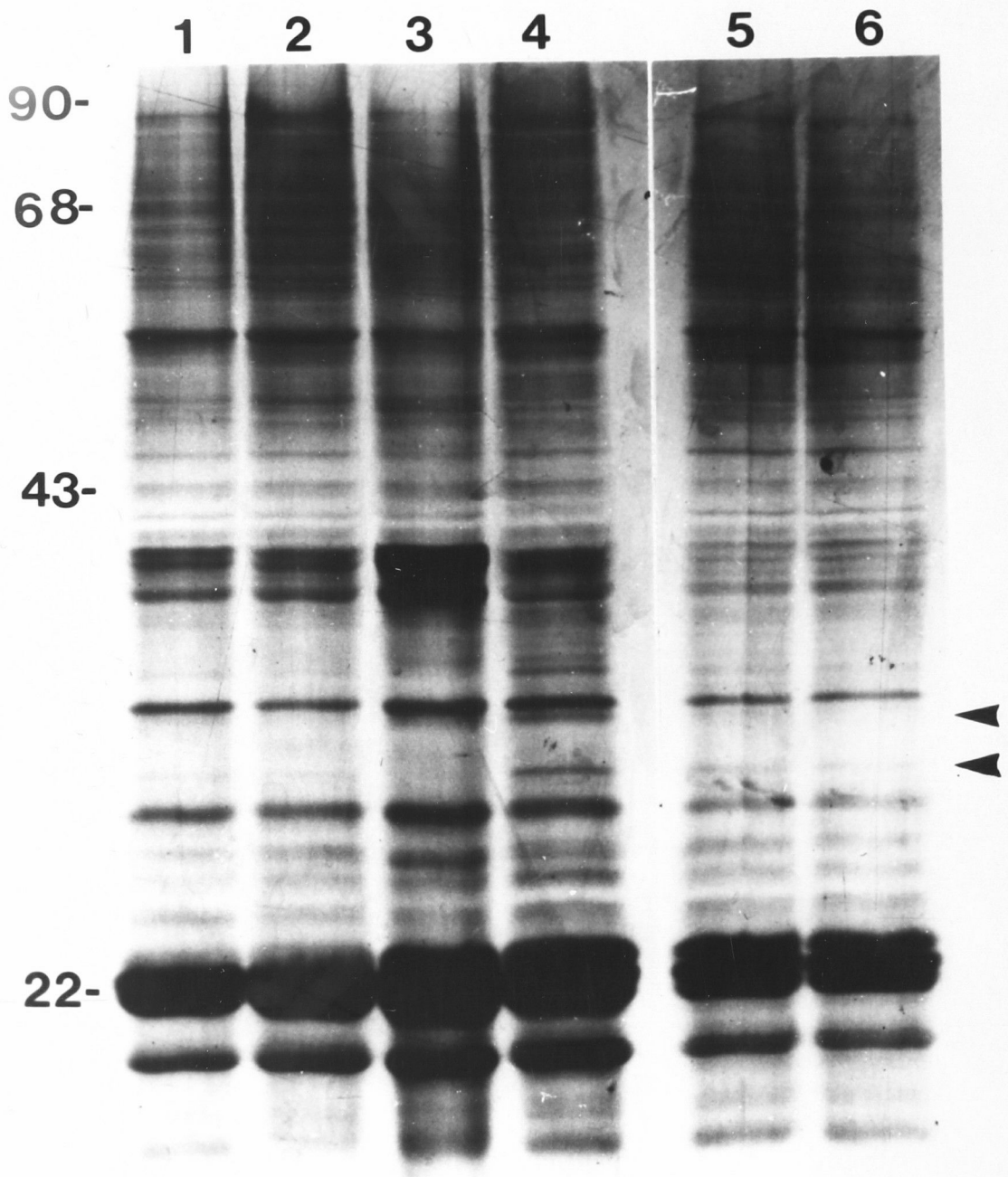

MOLECULAR WEIGHT (X 10^{-3})
80
60
40
20
0.1
0.3
0.5
0.7
0.9
RELATIVE MOBILITY (Rm)

Figure 4. 0.1% SDS-12% polyacrylamide gel of 3T3-L1 (Lanes 1-4) and 3T3-C2 (Lanes 5 and 6) chromatin proteins (10μg per lane) extracted from cells at different time points. Lane: (1) 3T3-L1 chromatin proteins obtained from preconfluent cells; (2) 3T3-L1 chromatin proteins just prior to MIX, DEX, and insulin treatment (zero hours); (3) 3T3-L1 chromatin proteins after 12 hours of MIX, DEX, and insulin treatment; (4) 3T3-L1 chromatin proteins 90 hours after initial treatment with MIX, DEX, and insulin; (5) 3T3-C2 chromatin proteins before MIX, DEX, and insulin treatment; (6) 3T3-C2 chromatin proteins 90 hours after initial MIX, DEX, and insulin treatment. Molecular weights ($x10^{-3}$) from standards are shown. Apparent changes are noted with arrows.


1
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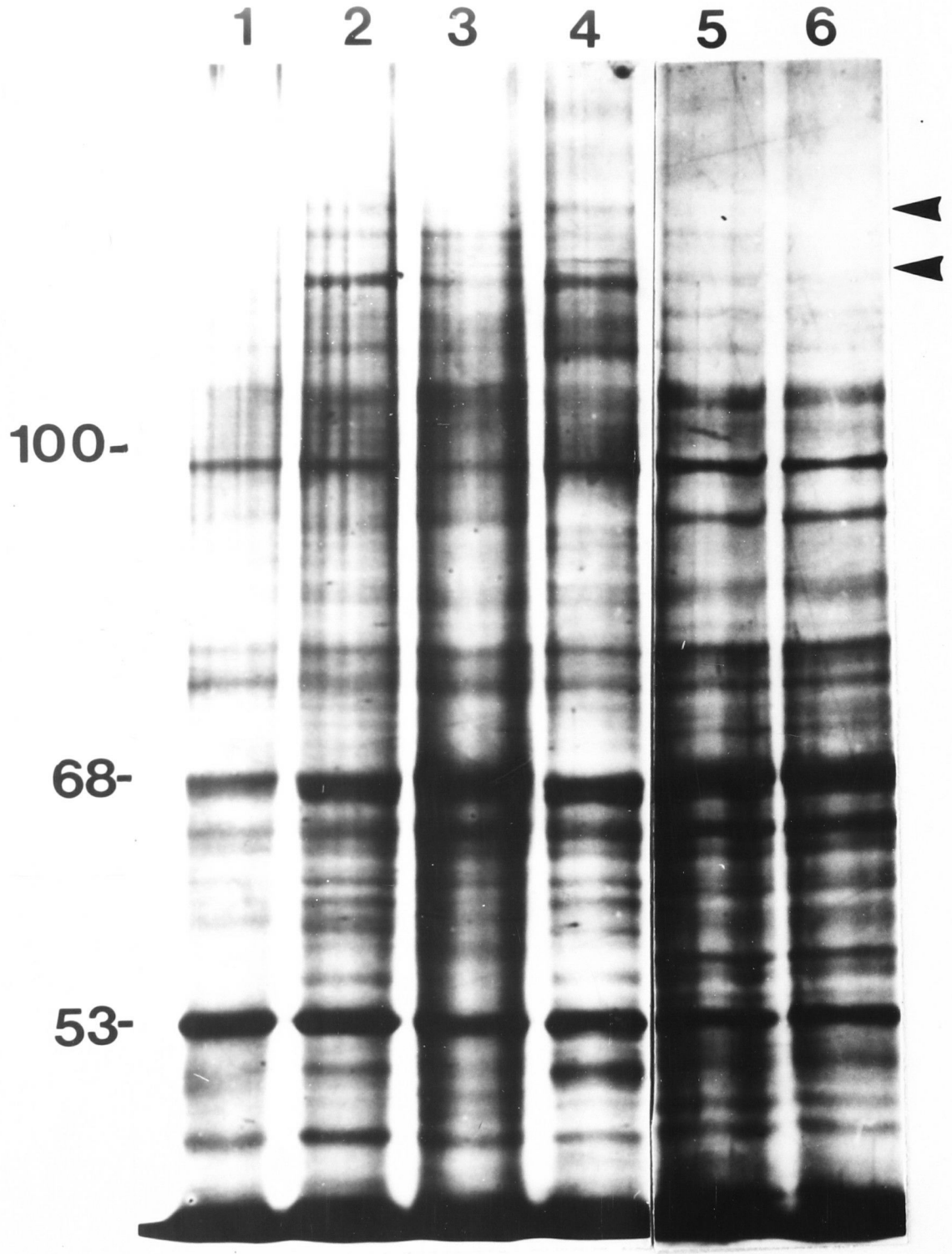
90-
68-
43-
22-

Figure 5. 0.1% SDS-7% polyacrylamide gel of 3T3-L1 (Lanes 1-4) and 3T3-C2 (Lanes 5 and 6) chromatin proteins (10 μg per lane) extracted from cells at different time points. Lane: (1) 3T3-L1 chromatin proteins just prior to MIX, DEX, and insulin treatment (zero hours); (2) 3T3-L1 chromatin proteins after 12 hours of MIX, DEX, and insulin treatment; (3) 3T3-L1 chromatin proteins after 24 hours of MIX, DEX, and insulin treatment; (4) 3T3-L1 chromatin proteins 90 hours after initial treatment with MIX, DEX, and insulin; (5) 3T3-C2 chromatin proteins 90 hours after initial MIX, DEX, and insulin treatment. Molecular weights ($x10^{-3}$) from standards are shown. Apparent changes are noted with arrows.


1
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3
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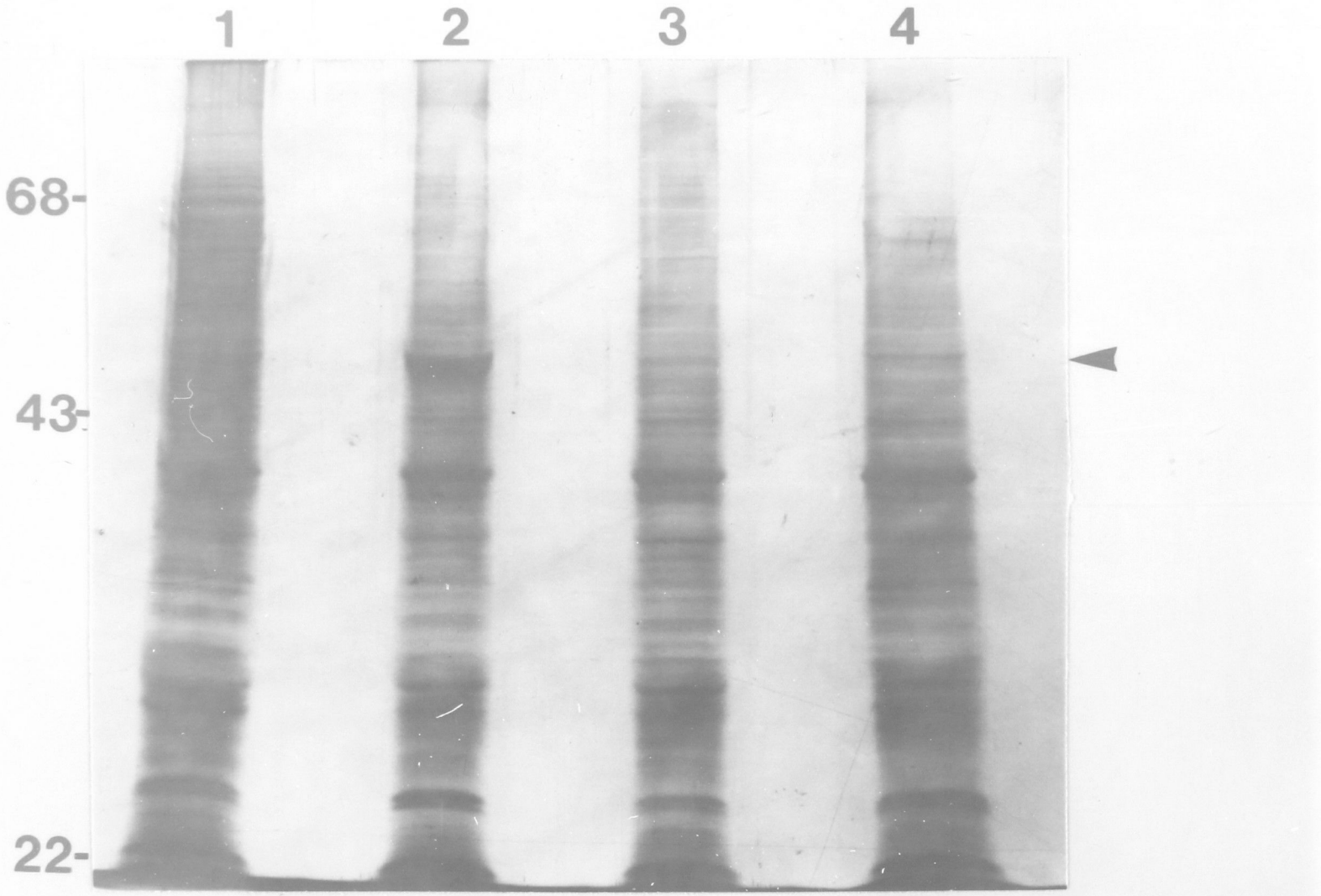
100-
68-
53-

Figure 6. 0.1% SDS-12% polyacrylamide gel of 3T3-L1 chromatin proteins (12 μg protein per lane) extracted at 4 different time points. Lane: (1) just prior to MIX, DEX, and insulin treatment; (2) after 12 hours of MIX, DEX, and insulin treatment; (3) after 24 hours of MIX, DEX, and insulin treatment; (4) 90 hours after initial treatment. Molecular weights ($x10^{-3}$) changes are noted with arrows.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3
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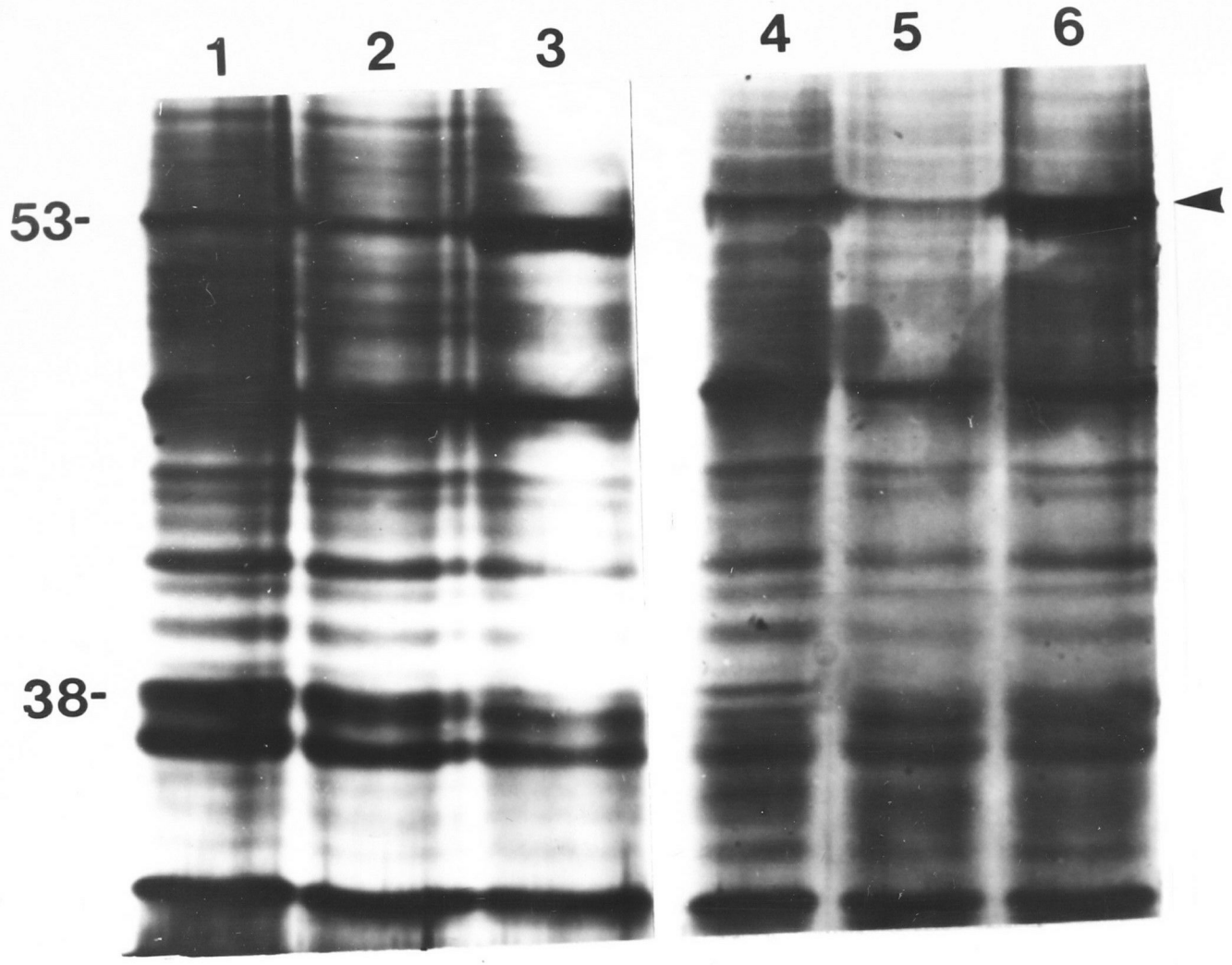
68-
43-
22-

Figure 7. 12% polyacrylamide gel of 3T3-L1 chromatin proteins (20 μg per lane) extracted at different time points. Lane: (1) just prior to MIX, DEX, and insulin treatment (zero hours); (2) after 12 hours of MIX, DEX, and insulin treatment; (3) after 24 hours of MIX, DEX, and insulin treatment; (4) 36 hours after initial treatment; (5) 90 hours after initial treatment. Molecular weights ($x10^{-3}$) are shown. Apparent changes are noted with arrows.


1
2
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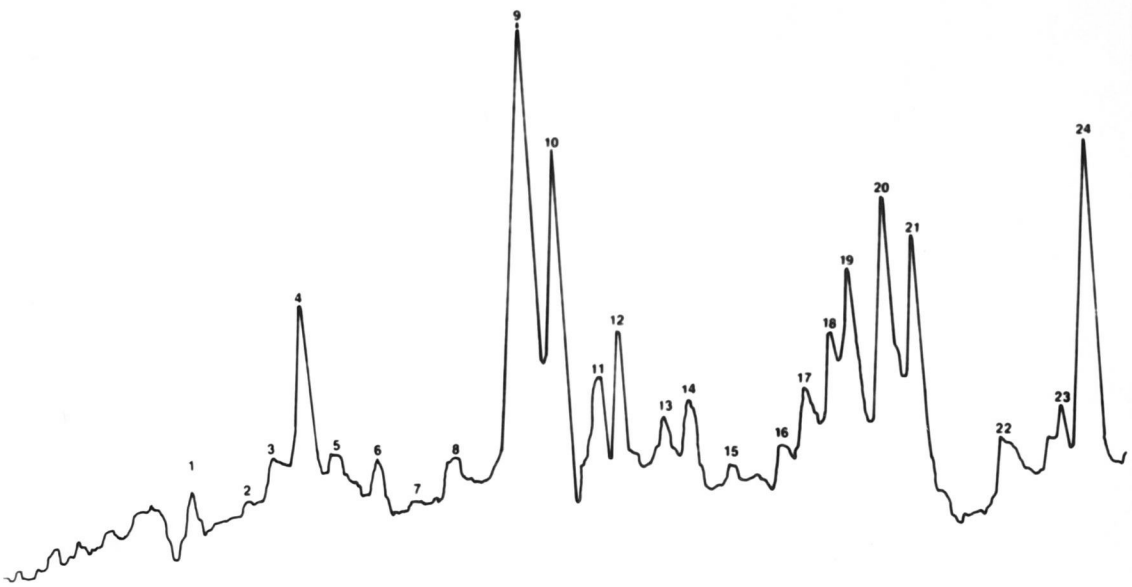
4
5
6
53-
38-

Figure 8. Autoradiogram densitometer scan of [3,4-^{3}H] leucine labelled chromatin proteins extracted from cells prior to treatment with MIX, DEX, and insulin.

Figure 9. Autoradiogram densitometer scan of [3,4-^{3}H] leucine labelled chromatin proteins extracted from cells 90 hours after initial treatment with MIX, DEX, and insulin. Arrow shows peak not found in scan of undifferentiated cell chromatin.

8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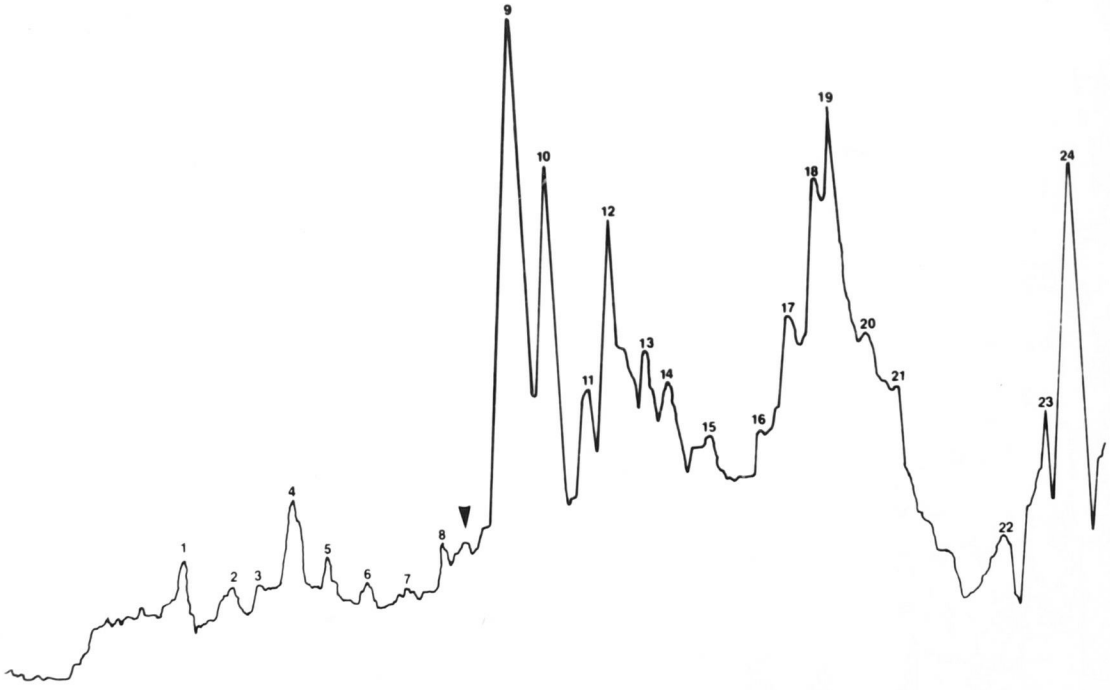
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Figure 10. Photograph of silver stained 6-15% polyacrylamide gradient gel containing [3,4-^{3}H] leucine labelled 3T3-L1 chromatin proteins. Lane (1) and (2) equal counts (2.5×10^{4} c.p.m.) of labelled chromatin proteins prior to treatment with MIX, DEX, and insulin (Lane 1) and 90 hours after treatment (Lane 2). Molecular weights from standards ($\times 10^{-3}$) are shown. Changes are noted with arrows.

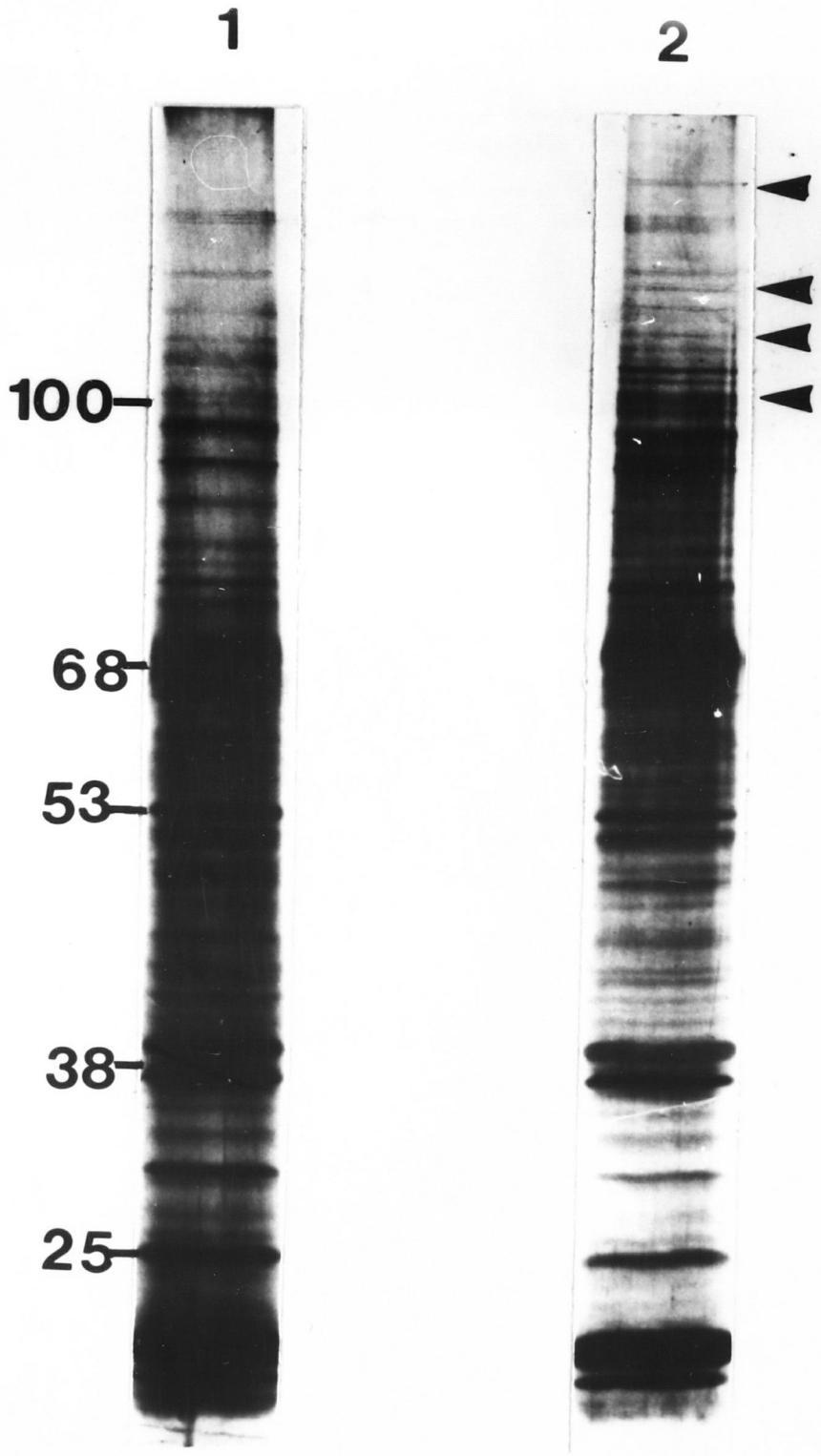
1
2
100
68
53
38
25

TABLE 1.

PEAKS FROM DENSITOMETER SCAN OF [3,4^{3}H] LEUCINE LABELLED CHROMATIN PROTEIN AUTORADIOGRAM

Peak Number From Figures 8 and 9	Molecular Weight (Thousands)	Amount of Label Incorporated Into Protein in Differentiated Compared to Undifferentiated Cell Chromatin
1	130	Increased
2	110	Increased
3	100	Decreased
4	95	Decreased
5	85	Unchanged
6	80	Decreased
7	75	Increased
8	73	Decreased
9	68	Unchanged
10	66	Unchanged
11	60	Unchanged
12	53	Increased
13	50	Increased
14	48	Increased
15	45	Increased
16	44	Unchanged
17	43	Increased
18	41	Increased
19	38	Increased
20	35	Decreased
21	33	Decreased
22	30	Unchanged
23	27	Increased
24	25	Unchanged

DISCUSSION

The methods used to isolate and separate chromatin proteins from intact cells allows the identification of at least 70 individual protein bands. The proteins vary in molecular weight from 18,000 to over 200,000 daltons. (Large amounts of protein exist in the 20,000 daltons range corresponding to the histones.) Other proteins which appear in comparably larger amounts include proteins with molecular weights of 68,000; 13,000; 38,000; and 25,000. One protein band of 53,000 daltons is seen to fluctuate in amount over different time intervals. This increased amount was seen in one instance after 12 hours post MIX, DEX, and insulin treatment and then again after 36 and 90 hours. A similar fluctuation in a protein of 45,000-55,000 daltons has been noted by other investigators (Bonner, 1975; Douvas, 1975). The pattern of fluctuation observed here suggests that the increased amount of protein is in response to some cyclic process such as cell division. This could possibly be a protein such as actin or tubulin involved in the mitotic process (Douvas, 1975).

Several new protein bands were seen to arise after cells began to differentiate. Among these were proteins with approximate molecular weights of 30,000; 70,000; 110,000; 150,000; and 200,000 daltons. These changes were not observed in the "undifferentiating" 3T3-C2 cell line explosed to the same chemical treatment that induces differentiation in 3T3-L1 cells. This fact suggests that these non-histone proteins are associated with the differentiation process and can be considered candidates for regulatory proteins.

Many investigators using different models (Keppel et al., 1977;

Sidhu, 1979; Heizman et al., 1980; Perle and Neuman, 1980) have made similar claims of a wide range of proteins but have not had access to or have not used a control cell line from which to compare results of experimental conditions. Therefore, in many cases, claims made that changes in particular proteins are differentiation induced or these proteins are regulators of differentiation are claims that are not sufficiently supported. One source of error in this type of study is contamination from cytosolic proteins. This is a sizable problem yet most investigators fail to show evidence that contamination of their chromatin proteins has been minimized. It can be concluded from this that many reports which show large number of changes in NHC proteins during differentiation and overall large number of chromatin proteins could have a degree of cytosolic contamination. The process used to extract chromatin proteins from cells in this study does so with a verificably negligible amount of contamination from cytosolic proteins. This fact could account for the low number of chromatin proteins represented and few changes seen compared with other studies (Peterson and McConkey, 1976; Sidhu, 1979; Lewin, 1980; LeParco et al., 1981). The possibility that chromatin proteins exist in smaller numbers has been supported by recent evidence that limits the number to well under 200 in eukaryotic cells (Personal Communication, Dr. James Paulson).

The production of many NHC proteins is increased and some decreased as a cell moves toward full differentiation. This has been evidenced in several other cell lines (Rodriguez et al., 1979; Heizman et al., 1980, LeParco, 1981; Morioka et al., 1981) and was demonstrated here in the [3,4^{3}H] leucine labelled chromatin protein study. However,

the same trend was not seen in every silver stained gel. This was because of the difficulty in adding equal amounts of protein to each lane of a gel prior to electrophoresis. The concentration of protein applied to each gel lane was very small (5-20 μg) and it proved difficult to accurately measure the concentration in a sample prior to its addition to the gel lane. On the other hand, adding equal counts of [3,4-^{3}H] leucine labelled proteins proved to be considerably easier resulting in a more dependable comparison of differences. The silver staining method employed in this study was useful in providing a high resolution look at overall features and changes in chromatin proteins. The method proved both simple and economical requiring only nanogram quantities of protein for adequate resolution.

This study describes the overall characteristics of 3T3-L1 chromatin proteins and shows how these proteins exhibit some degree of variation during differentiation. In order to get a more complete picture of these proteins it would be best to perform a variety of further studies. First, it would be important to perform a two-dimensional electrophoresis study with isoelectric focusing in the first dimension (separation based on charge) followed by SDS-polyacrylamide gel electrophoresis in the second dimension (separation based on size). This method would resolve a much larger number of proteins and would permit the resolution of proteins with the same molecular weight but different charge properties. This higher resolution offered by two-dimensional electrophoresis could offer more insight into the problem. It would also be profitable to look at different fractions of NHC proteins by selectively eluting proteins from chromatin. This could be accomplished by extracting proteins with

buffers of increasing ionic strength. Using the method developed by Chiu et al. (1977) it has been shown that three fractions can be obtained from chromatin in this manner. The loosely bound non-histone group, the histone group, and the tightly bound non-histone group could each be obtained and subjected to both one and two-dimensional electrophoresis. In looking for a possible regulatory protein, this would be advantageous as any such protein would be most likely in close association and tightly bound to DNA. Finally, it would be interesting to look at the high mobility group (HMG) proteins by extracting them from chromatin with perchloric acid and subjecting them to electrophoresis on acetic acid-urea gels (Seyedin et al., 1981). Comparisons between differentiated and undifferentiated cell groups and the results compared to other systems such as Friends' erythroleukemia cells could be useful.

In conclusion, the significance of changes observed in the populations of NHC proteins during differentiation is open to speculation. Can there in fact be proteins which repress or promote the expression of genetic material or are these observed changes the result of new gene expression? The latter probably holds true because of the myriad of functions different protein molecules have in the eukaryotic nucleus. But what of actual regulation of gene expression? The evidence presented in the literature suggests that there can be specific protein regulators in eukaryotic cells. There are, however, other possibilities. One such is described in a model proposed by Lavett (1984). In this model based on the structure of the beta and gamma globin gene, RNA transcripts from the introns of beta family genes bind to the promotor of the gamma globin gene causing a change in the secondary structure and preventing transcription

of the gene. In many ways this type of regulation would seem more economical for the cell. A transcript would be more energy efficient and direct rather than first making an RNA transcript which then must be processed and translated to protein which then returns to the nucleus as a regulatory element. However, gene regulation by protein promoter-inducer-repressor relationships occur in prokaryotes and evidence presented in eukaryotic systems suggest this possibility. Perhaps the best way to approach this problem is through studies at the molecular level employing model systems such as the 3T3-L1 preadipocyte.

REFERENCES

Adolph, K. W., S. M. Cheng, and U. K. Laemmli. 1977. Role of non-histone proteins in metaphase chromosome structure. Cell. 12: 805-816.

Adolph, K. W. and J. P. Phelps. 1982. Role of non-histones in chromosome structure. J. Biol. Chem. 257: 9086 - 9092.

Balhorn, R., D. Jackson, D. Granner, and R. Chalkly. 1975. Phosphorylation of the lysine rich histones throughout the cell cycle. Biochemistry. 14: 2504 - 2511.

Bedi, K. S. and D. J. Goldstein. 1976. Apparent anormalies in nuclear Feulgen-DNA contents. Role of systematic microdensitometric errors. J. Cell Biol. 71: 68-88.

Bidney, D. L. and G. R. Reeck. 1978. Purification from cultured hepatoma cells of two nonhistone chromatin proteins with preferential affinity for single stranded DNA: apparent analogy with calf thymus HMG proteins. Biochem. Biophys. Res. Commun. 86: 1211-1220.

Bonner, W. M. 1975. Protein migration into nuclei. I. Frog oocyte nuclei in vivo accumulate microingected histones, allow entry to small proteins, and exclude large proteins. J. Cell. Biol. 64: 421-430.

Bradbury, E. M. 1982. DNA, Chromatin and Chromosomes. New York: John Wiley and Sons.

Bustin, M. and R. D. Cole. 1969. A study of the multiplicity of lysine rich histones. J. Biol. Chem. 244: 5286-5290.

Cartwright, I. L., S. M. Abmayr, G. Fleishmann, K. Lowenhaupt, C. R. Elgin, M. A. Keene, and G. C. Howard. 1982. Chromatin structure and gene activity: the role of non-histone chromosomal proteins. Crit. Rev. Biochem. 13: 1-85.

Chen, Z., J. Banks, A. Rifkind and P. A. Marks. 1982. Inducer-mediated commitment of murine erythroleukemia cells to differentiation: a multistep process. Proc. Natl. Acad. Sci. USA. 79: 471-475.

Chiu, J., H. Fujitani, and S. Hnilica. 1977. Methods for selective extraction of chromosomal nonhistone proteins, p. 283-296. In G. Stein and J. Stein (eds.) Methods in Cell Biology Volume XVI. New York: Academic Press.

Coleman, R. A., B. C. Reed, J. C. Mackall, A. K. Student, and M. D. Lane. 1978. Selective changes in microsomal enzymes of triacylglycerol phophatidylcholine and phosphatidylethanolamine during differentiation of 3T3-L1 readipocytes. J. Biol. Chem. 253: 7256-7259.

DeLange, R. J. and E. L. Smith. 1971. Histones: structure and function. Ann Rev. Biochem. 40: 279-314.

DeRobertis, E. D. P. and E. M. F. DeRobertis. 1980. Cell and Molecular Biology. Philadelphia: Saunders College.

Diaz, M. O., G. Barsacchi-Pilone, K. A. Mahon, and J. G. Gall. 1981. Transcripts from both strands of a satellite DNA occur on lampbrush loops of the newt Notopthalalmus. Cell. 24: 649-653.

Douvas, A. S., C. A. Harrington, and J. Bonner. 1975. Major nonhistone proteins of rat liver chromatin: preliminary identification of myosin, actin, tubulin, and tropomyosin. Proc. Nat. Acad. Sci. USA. 72: 3902-3906.

DuPraw, E. J. 1965. Macromolecular organization of nuclei and chromosomes: a folded fiber model based on whole mount electron microscopy. Nature. 206: 338-343.

Elgin, S. C. R. and L. E. Hood. 1973. Chromosomal proteins of Drosophila embryos. Biochemistry. 11: 772-781.

Finch, J. T. and A. Klug. 1976. Solenoidal model for superstructure in chromatin. Proc. Natl. Acad. Sci. USA. 73: 1897-1899.

Finch, J. T., L. C. Lutter, D. Rhodes, R. S. Brown, B. Rushton, M. Levitt, and A. Klug. 1977. Structure of nucleosome core particles of chromatin. Nature. 269: 29-35.

Freese, E. 1958. The arrangement of DNA in the chromosome. Cold Spring Harbor Symp. Quant. Biol. 23: 13-18.

Georgieva, E. I., I. G. Pasher, and R. G. Tsanev. 1981. Distribution of high mobility group and other acid soluble proteins in fractionated chromatin. Biochem. Biophys. Acta. 652: 240-245.

Goodwin, H. G., C. G. P. Mathew, C. A. Wright, C. D. Venkov, and E. W. Johns. 1979. Analysis of the high mobility group proteins associated with salt-soluble nucleosomes. Nucl. Acids Res. 7: 18-24.

Goodwin, G. H. and C. G. P. Mathew. 1982. Role in gene structure and function. In E. W. Johns (ed.) The HMG Chromosomal Proteins. New York: Academic Press.

Goodwin, G. H., C. Sanders, and E. W. Johns. 1973. A new group of chromatin associated proteins with a high content of acidic and basic amino acids. Eur. J. Biochem. 38: 14-19.

Green, H. and O. Kehinde. 1974. Sublines of mouse 3T3 cells that accumulate lipid. Cell. 1: 113-116.

Green, H. and O. Kehinde. 1975. An established preadipose cell line and it differentiation in culture II. Factors affecting the adipose conversion. Cell. 5: 19-27.

Green, H. and O. Kehinde. 1976. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell. 7: 105-113.

Green, H. And M. Meuth. 1974. An established pre-adipose cell line and its differentiation in culture. Cell. 3: 127-133.

Gurley, L. R., R. A. Walters, and R. A. Tobey. 1974. Cell cycle specific changes in histone phosphorylation associated with cell proliferation and chromosome condensation. J. Cell. Biol. 60: 356-364.

Heizmann, C. W., E. M. Arnold, and C. C. Kuenzle. 1980. Fluctuations of non-histone chromosomal proteins in differentiating brain cortex and cerebellar neurons. J. Biol. Chem. 255: 11504-11511.

Hozier, J., M. Renz, and P. Nehls. 1977. The chromosome fiber: evidence for an ordered superstructure of nucleosomes. Chromosoma. 62: 301-303.

Igo-Kemenes, T., W. Horz, and H. G. Zachau. 1982. Chromatin. Ann. Rev. Biochem. 51: 89-121.

Igo-Kemenes, T. and H. G. Zachau. Domans in chromatin structure. Cold Spring Harbor Symp. Quant. Biol. 42: 109-115.

Isenberg, I. 1979. Histones. Ann. Rev. Biochem. 48: 159-191.

Jackson, J. B. and R. L. Rill. 1981. Circular dichroism, thernal denaturation, and deoxyribonuclease I digestion studies of nucleosomes highly enriched in high mobility group protein HMG1 and HMG2. Biochemistry. 20: 1042-1054.

Jacob, F. and J. Monod. 1961. Genetic regulartory mechanisms in the synthesis of proteins. J. Mol. Biol. 3: 318-356.

Javaherian, K., M. Sadeghi, and L. F. Liu. 1979. Nonhistone proteins HMG1 and HMG2 unwind DNA double helix. Nucl. Acids. Res. 6: 3569-3572.

Johns, E. W. 1982. The HMG Chromosomal Proteins. New York: Academic Press.

Kamiyama, M. and T. Y. Wang. 1971. Activated transcription from rat liver chromatin by non-histone proteins. Biochem. Biophys. Acta. 228: 563-576.

Keppel, F. B. Allet, and H. Eisen. 1977. Appearance of a chromatin protein during the erythroid differentiation of Friend virus-transformed cells. Proc. Natl. Acad. Sci. USA. 54: 653-656.

Kinkade, J. M. and R. D. Cole. 1966. A structural comparison of different lysine rich histones of calf thymus. J. Biol. Chem. 241: 5798-5805.

Kornberg, R. D. and J. O. Thomas. 1974. Chromatin structure: oligomers of the histones. Science. 184: 865-873.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 222: 680.

Lake, R. S. and N. P. Salzman. 1972. Occurrence and properties of chromatin associated H1 histone phosphokinase in mitotic Chinese hamster cells. Biochemistry. 11: 4817-4825.

Lake, R. S. 1973. H1 histone phosphorylation in metaphase chromosomes of cultured Chinese hamster cells. Nature New Biol. 242: 145-146.

Lavett, D. K. 1984. Secondary structure and intron-promoter honology in globin-switching. Am. J. Hum. Genet. 36: 338-345.

Lee, K. S., M. Handelkern, and D. M. Crothers. 1981. Solution structured studies of chromatin fibers. Biochemistry. 20: 1438-1446.

LeParco, Y. M. Fontes, and Y. Thouvey. 1981. Variations of chromatin-associated proteins during the prereplicative phase of traumatic regeneration of the Polychalts Annelid Owinia fusiformis. Cell Differentiation. 10: 253-265.

Lewin, B. 1980. Gene Expression. New York: John Wiley and Sons.

Mackall, J. M. Meredith, and M. D. Lane. 1979. A mild procedure for the rapid release of cytoplasmic enzymes from cultured animal cells. Anal. Biochem. 95: 270-274.

Mackall, J. C. A. Student, S. Polakis, and M. D. Lane. 1976. Induction of lipogenesis during differentiation in a preadipocyte cell line. J. Biol. Chem. 251: 6462-6464.

Mardian, J. K. W., A. E. Paton, G. J. Bunick, and D. E. Olins. 1980. Nucleosome cores have two specific binding sites for nonhistone chromosomal proteins HMG14 and HMG17. Science. 209: 1535-1539.

Marks, D. B., W. K. Paik, and T. W. Borun. 1973. The relationship of histone phosphorylation to deoxyribonucleic acid replication and mitosis during the HeLa S-3 cell cycle. J. Biol. Chem. 248: 5660-5667.

Merril, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science. 211: 1437-1438.

Mirsky, A. E. and H. Ris. 1951. The composition and structure of isolated chromosomes. J. Gen. Physiol. 34: 475-492.

Mirsky, A. E. and H. Rs. 1952. The DNA content of animal cells and its evolutionary significance. J. Gen. Physiol. 34: 451-462.

Morioka, K., K. Tanaka, and T. ONO. 1981. Comparison of nuclear proteins of differentiation-induced and uninduced Friend erythroleukemia cells. J. Biochem. 89: 1633-1638.

Morris, N. R. 1976. Nucleosome structure in Aspergillus nidulans. Cell. 8: 357-364.

Nakane, M., T. Ide, K. Anzai, S. Ohara, and T. Andoh. 1978. Supercoiled DNA folded by nonhistone proteins in cultured mouse carcinoma cells. J. Biochem. 84: 145-157.

Nishizuka, Y., K. Ueda, K. Nakazawa, and O. Hayaishi. 1967. Studies on the polymer of Adenosine diphosphate ribose. J. Biol. Chem. 242: 3164-3171.

Noll, M. 1974. Subunit structure of chromatin. Nature. 251: 249-264.

Noll, M. and R. D. Kornberg. 1977. Action of micrococcal nuclease on chromatin and the location of H1. J. Mol. Biol. 109: 393-404.

Norman, G. L. and I. Bekhor. 1981. Enrichment of selected active human gene sequences in the placental deoxyribonucleic acid fraction associated with tightly bound nonhistone chromosomal proteins. Biochemistry. 20: 3568-3578.

O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell. 12: 1133-1142.

Olins, A. L. and D. E. Olins. 1974. Sheroid chromatin units (v bodies). Science. 183: 330-334.

Ord, M. G. and L. A. Stocken. 1977. Adenosine diphosphate ribosylated histones. Biochem. J. 161: 183-192.

Patthy, L., E. L. Smith, and J. Johnson. 1973. Histone III. V. The amino acid sequence of pla embryo histone III. J. Biol. Chem. 248: 6834-6840.

Perle, M. A., C. M. Leonard, and S. A. Neuman. 1982. Developmentally regulated nonhistone proteins: Evidence for deoxyribonucleic acid binding role and localization near deoxyribonuclease I sensitive domains of precartilage cell chromatin. Biochemistry. 21: 2378-2386.

Perle, M. A. and S. A. Newman. 1980. talpid^2 mutant of the chicken with perturbed cartilage development has an altered precartilage-specific chromatin protein. Proc. Natl. Acad. Sci. USA. 77: 4828-4830.

Platz, R. D. and L. S. Hnilica. 1973. Phosphorylation of nonhistone chromatin proteins during sea urchin development. Biochem. Biophys. Res. Commun. 54: 222-227.

Peterson, J. L. and E. H. McConkey. 1976. Nonhistone chromosomal proteins from HeLa cells. A survey by high resolution, two dimensional electrophoresis. J. Biol. Chem. 251: 548-554.

Rall, S. C. and R. D. Cole. 1971. Amino acid sequence and sequence variability of the amino terminal regions of lipine rich histones. J. Biol. Chem. 246: 7175-7190.

Ris, H. and B. L. Chandler. 1963. The ultrastructure of genetic systems in procaryotes and eukaryotes. Cold Spring Harbor Symp. Quant. Biol. 28: 1-8.

Ris, H., and J. Korenberg. 1979. Chromosome structure and levels of chromosome organization in Cell Biology: A Comprehensive Treatise. New York: Academic Press.

Rodriguez, L. B., K. K. Klein, M. Amoruso, and F. F. Becker. 1979. Analysis of chromosomal proteins of fractionated chromatin from rat liver and transplantable hepatocellular carcinomas. Int. J. Cancer. 24: 490-497.

Rubin, C. S., A. Hirsch, C. Fung, and O. M. Rosen. 1978. Development of hormone receptors and hormone responsiveness in vitro. J. Biol. Chem. 253: 7570-7578.

Sanders, L. A. 1974. Isolation and characterization of the nonhistone chromosomal proteins from developing avian erythroid cells. Biochemistry. 13: 527-534.

Seyedin, S. M., J. R. Pehrson, and R. D. Cole. 1981. Loss of chromosomal high mobility group proteins HMG1 and HMG2 when mouse neuroblastoma and Friend erythroleukemia cells become committed to differentiation. 1981. Proc. Natl. Acad. Sci. USA. 78: 5988-5992.

Sidhu, R. 1979. Two dimensional electrophoretic analysis of proteins synthesized during differentiation of 3T3-L1 preadipocytes. J. Biol. Chem. 254: 11111-11118.

Smith, J. A. and L. A. Stocken. 1973. Identification of poly (ADP-ribose) covalently bound to histone F1 *in vitro*. Biochem. Biophys. Res. Commun. 54: 297-300.

Sperling, L., and A. Klug. 1977. X-ray studies on "native" chromatin. J. Mol. Biol. 112: 253-259.

Stalder, J., M. Groudine, J. B. Dodgson, J. D. Engel, and H. Weintraub. 1980. Hb switching in chickens. Cell. 19: 973-975.

Stedman, E. and E. Stedman. 1950. Cell specificity of histones. Nature. 166: 780-781.

Stephenson, E. G., H. P. Erba, and J. C. Gall. 1981. Histone gene clusters of the newt *Notopthalmus* are separated by long tracts of satellite DNA. Cell. 24: 639-642.

Suau, P., E. M. Bradbury, and J. P. Baldwin. 1979. Higher-order structures of chromatin in solution. Eur. J. Biochem. 97: 593-602.

Teng, C. S., C. T. Teng, and V. G. Allfrey. 1971. Studies of nuclear acidic proteins. Evidence for their phosphorylation, tissue specificity, selective binding to DNA and stimulatory effects on transcription. J. Biol. Chem. 246: 3957-3609.

Thomas, J. O. and P. J. Butler. 1980. Size dependence of a stable higher-order structure of chromatin. J. Mol. Biol. 144: 89-94.

Todaro, G. J. and H. Green. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. 17: 299-302.

Ueda, K., H. OKayama, M. Fukushima, and O. Hayaishi. 1977. Seminar on poly (ADP-ribose) and ADP-ribosylation of protein. 77: 1 - 11.

Weisbrod, S. and H. Weintraub. 1979. Isolation of a subclass of nuclear proteins responsible for conferring a DNase I-sensitive structure on globin chromatin. Proc. Natl. Acad. Sci. USA. 76: 630-640.