On the Chromatin Structure of the Amplified, Transcriptionally Active Gene for Dihydrofolate Reductase in Mouse Cells*

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The method for two-dimensional hybridization mapping of nucleosomes (Levinger, L., Barsoum, J., and Varshavsky, A. (1981) J. Mol. Biol. 146, 287-304) was used to analyze chromatin structure of the gene for dihydrofolate reductase (DHFR reductase; 5,6,7,8-tetrahydrofolate:NADP+ oxidoreductase (EC 1.5.1.3)) in L6178Y-R mouse cells. The DHF reductase gene in these cells is amplified about 350-fold as a result of selection for resistance to methotrexate. Dramatic overproduction of DHF reductase mRNA in L6178Y-R cells suggests that most of the DHF reductase genes in these cells are transcribed.

We report that all major mononucleosomal species resolvable by two-dimensional fractionation are detected by both DHF reductase- and satellite DNA-specific hybridization probes. Although the DHF reductase and satellite hybridization patterns differ somewhat from each other and from the total mononucleosomal pattern, their overall similarity is very high. In particular, no large differences in the abundance of mononucleosomes containing high mobility group non-histone proteins (HMG) 14 and 17 are seen between the DHF reductase and satellite chromatin regions under a wide variety of conditions for chromatin isolation, digestion, and fractionation. Possible interpretations of the apparent lack of selectivity of HMG-chromatin interactions in this system are discussed.

We also found that the amplified DHF reductase genes possess a wide range of nucleosomal repeat lengths close to that in the bulk chromatin. In contrast, the range of nucleosomal repeat lengths in the satellite chromatin is much narrower than in both DHF reductase and bulk chromatin.

Most of the chromatin in a eukaryotic cell is organized into nucleosomal fibers (for a review see Kornberg, 1981). Both compositional and conformational heterogeneity along the fibers are observed (for reviews see McGhee and Felsenfeld, 1980; Mathis et al., 1980). Chromatin functions including replication, transcription, and mitotic compaction might be expected to require the non-random distribution of minor chromosomal proteins and/or specific modifications of major proteins, such as histones, at different moments of the cell cycle and in different regions of the genome. In particular, transcriptionally active chromatin regions were shown to differ significantly from the bulk of nontranscribed chromatin in their susceptibility to nucleases, solubility, and other parameters (Weintraub and Groudine, 1976; Garel and Axel, 1976; Levy-Wilson et al., 1979; Bakayev et al., 1979). Since these features of active chromatin are certainly not confined to promoter regions only (Weintraub and Groudine, 1976; Panet and Cedar, 1977), there should exist distinct compositional and/or conformational differences between transcribed and nontranscribed chromatin fibers.

The better defined compositional differences between transcribed and nontranscribed chromatin discovered so far is the preferential association of the non-histone proteins, HMG14 and HMG17 (Walker et al., 1977; Vidali et al., 1977; Isackson and Reeck, 1981), with the transcribed chromatin isolated from terminally differentiating cells, in particular, from chicken erythroblasts (Weisbrod et al., 1980; Gazit et al., 1980). Purified HMG14 and -17 preferentially bind in vitro to globin DNA-containing mononucleosomes from chicken erythroblasts (Weisbrod and Weintraub, 1981; Sandeen et al., 1980; see also Mardian et al., 1980). Structural features of transcribed, globin DNA-containing nucleosomes that determine their preferential binding of HMGs remain unknown, although a correlation exists with undermethylation of transcribed DNA (Mandel and Chambon, 1978; Van der Ploeg and Flavell, 1980; Shen and Maniatis, 1980; Groudine et al., 1981).

We have recently developed a new approach to the analysis of associations of variant nucleosomes with specific DNA sequences (Levinger et al., 1981). The approach consists of two-dimensional fractionation of nucleosomes (low ionic strength electrophoresis of deoxyribonucleoproteins in the first dimension followed by DNA electrophoresis in the second dimension), with subsequent electrophoretic transfer of nucleosomal DNA to DBM paper for hybridization with specific DNA probes. Positions of DNA spots in a two-dimensional DNP-DNA pattern are a function of nucleosome composition and/or conformation in the first (DNP) dimension. Protein composition of separated nucleosomes can be determined by running parallel second dimension protein gels. This method therefore enables one to deduce composition of subsets of

*The abbreviations used are: HMG, high mobility group non-histone proteins; DHF reductase, dihydrofolate reductase; DBM paper, dianobenzyloxymethyl paper; PMSF, phenylmethylsulfonyl fluoride; TPCK, 1-1-tosylamide-2-phenylethyl chloromethyl ketone; TLCK, N⁰-p-tosyl-L-lysine chloromethyl ketone; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(b-o-aminoethyl ether)-N,N',N⁰,N⁰'-tetraacetic acid; SDS, sodium dodecyl sulfate; DNP, deoxyribonucleoproteins; hp, base pairs; kb, kilobase pairs; MN1, core mononucleosomes containing 146-bp DNA fragment, core histone octamer but neither histone H1 nor HMG proteins nor ubiquitin-H2A semihistone; MN1HMG, a subset of mononucleosomes that contain proteins HMG14 and -17; MN2, a metastable mononucleosomal intermediate containing from ≈160- to ≈180-bp-long DNA fragment, core histone octamer, and 1 molecule of histone H1.

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nucleosomes containing DNA sequences hybridizing to a specific probe. Application of this approach to the chromatin from HeLa cells (Levinger et al., 1981) has shown that highly repetitive, presumably transcriptionally inactive regions of HMG chromatin contain significant proportions of the total HMG14 and -17. A similar conclusion was reached by Mathew et al. (1981) using a different experimental approach. The presence of HMG proteins in heterochromatic, apparently transcriptionally inactive chromatin regions indicated that specific interactions of HMG proteins with transcribed genes, detected previously by other methods in other systems, are apparently superimposed over a significant background of non-specific HMG-chromatin interactions.

For further studies we turned to L5178Y-R mouse cells obtained by selection for resistance to high concentrations of methotrexate, a specific inhibitor of the key cellular enzyme, dihydrofolate reductase (Dolnick et al., 1979). The DHF reductase gene dosage in these cells is approximately 350 times higher than in parental, methotrexate-sensitive cells (Schimke et al., 1977; Dolnick et al., 1979; Birkenmeier, 1981). Dramatic overproduction of DHF reductase mRNA in L5178Y-R cells (see below) suggests that most of the DHF reductase genes in these cells are transcribed. Although DHF reductase mRNA and enzyme production in mouse cells has been shown to be highest in early S phase (Johnson et al., 1978), recent data by Kellens et al. indicate that this increase is a consequence of post-transcriptional control and that the DHF reductase gene is transcribed at an approximately constant rate throughout the cell cycle.

We analyzed the chromatin structure of the amplified DHF reductase gene using the two-dimensional hybridization mapping approach and report a lack of preferential binding of HMG14 and -17 to the DHF reductase chromatin regions as compared with both satellite-containing chromatin and total chromatin from the L5178Y-R cells. We discuss possible interpretations of these results which are not necessarily incompatible with the concept of preferential HMG binding to certain active genes.

**EXPERIMENTAL PROCEDURES**

_Cell Culture and in Vivo Labeling_—Suspension cultures of L5178Y/R3 cells (a gift from Dr. J. Bertino, Yale University Medical School, New Haven, CT) were propagated in plastic tissue culture flasks (Corning). In Facher's medium for leukemia cells of mice (Gibco) supplemented with penicillin/streptomycin and 10% calf serum (Gibco).

For DNA labeling of C3 cells, [3H]thyminde (New England Nuclear, 20 Ci/mmol) was added to a medium initial cell density of about 8 x 10^4 cells/ml which allowed exponential cell growth throughout the labeling period. Protein labeling of exponentially growing C3 cells was accomplished by the addition of [3H]lysine (New England Nuclear, 60 Ci/mmol) at 8 µCi/ml for approximately 24 h.

**Quantitation of DHF Reductase mRNAs**—Total cytoplasmic RNA was isolated from C3 and 3T6 cells by the method of Berger and Birkenmeier (1979). Poly(A)^+ RNA was purified by passing the total cytoplasmic RNA twice through oligo(dT)-cellulose columns (Berger and Cooper, 1975). RNA in separated poly(A)+ and poly(A)- fractions was precipitated with ethanol, resuspended in 1.5 mM g-tyosyl, 50% dimethyl sulfoxide and 10 mM Na-phosphate (pH 7.0), and incubated for 1 h at 10 °C (McMaster and Carmichael, 1977). After cooling the samples to room temperature, sucrose was added to 5% and the mixture was precipitated with ethanol, resuspended in 1.5 M glyoxal, 50% Na-phosphate (pH 7.0). Running buffer was the same and electrophoresis was at 3.5 V/cm for 12 h.

After electrophoresis, the gel was placed in 50 mM NaOH for 1 h at room temperature, followed by incubation at the same temperature in 2 µg/ml of ethidium bromide, 0.2 M Na-acetate (pH 4.0) for 30 min and then for an additional 15 min at 4 °C. After photographing RNA patterns using 366 nm UV illumination, RNA fragments were transferred from the gel to a DBM paper (Alwine et al., 1977) at 4 °C by blotting (Southern, 1975) using 0.2 M Na-acetate (pH 4.0) as the transfer buffer.

Cloned DHF reductase cDNA (pDHFR11, a gift from Dr. R. Schimke) labeled with [3H]lysine (Rigby et al., 1977) was hybridized to RNA on the DBM paper as described previously (Levinger et al., 1981) and autoradiography was performed at -70 °C using Kodak XAR-5 film and DuPont Lightning Plus intensifying screen.

**Quantitation of DHF Reductase Protein**—Approximately 3 x 10^6 of both 3T6 or C3 cells were pelleted and resuspended in 0.25 ml of 0.5% Nonidet P-40, 0.3 m sucrose, 1 mM phenylmethylsulfonyl fluoride (freshly added from 0.5 M stock in absolute ethanol), 3 mM CaCl_2, 10 mM Tris-HCl (pH 7.5). The suspension was vortexed to facilitate cell lysis and then centrifuged at 12,000 x g for 30 s. The supernatant (cytosol) and pellet (nuclei) were then analyzed by electrophoresis in 18% polyacrylamide-SDS gels (1.5 mm thick, 30 cm long) as described (Kellems and Kornberg, 1975). Proteins were stained with Coomassie brilliant blue R-250.

**Determination of DHF Reductase Gene Dosage**—A modified dot hybridization technique of Kafatos et al. (1979) was used as was previously described in detail (Varshavsky, 1981).

**Chromatin Preparation and Nuclease Digestion**—Different protocols were used.

1. Labeled C3 cells were centrifuged at 506 x g for 5 min at 4 °C, resuspended in 0.14 M NaCl, 5 mM Tris-HCl (pH 7.5), and centrifuged again. The pellet was resuspended in 0.5% Nonidet P-40, 5 mM Na-butyrat, 1 mM PMSF, 5 mM Na-EDTA, 10 mM Na-Hepes (pH 7.5) by gentle strokes in a loosely fitted Dounce homogenizer. After centrifugation at 2,000 x g for 5 min, the nuclear pellet was washed once in the lysin buffer and pellet again. This pellet was then washed twice for a total time of about 1 h in 0.1 mM PMSF, 0.1 mM CaCl_2, 1 mM Na-Hepes (pH 7.5) and finally resuspended in the same buffer containing 0.2 mM CaCl_2 to approximately 1 mg of DNA per ml. Digestion was carried out by the addition of staphylococcal nuclease (Sigma) to a final concentration of 5 µg/ml followed by incubation at 37 °C. Aliquots were taken at various points from 40 to 20 min digestion time and added to 0.5% volume of 50 mM Na-EDTA, 25 mM Na-EGTA (pH 7.5). Insoluble material was pelleted at 12,000 x g for 5 min. Supernatants were used immediately or frozen at -70 °C in the presence of 10% glycerol. Freezing and thawing did not influence the results obtained.

2. Chromatin was isolated and digested in the presence of divalent cations throughout the procedure. The method was that of Boyce et al., 1982 for the use of DTNB in isolation of SV40 viral nucleoprotein complexes. In addition, all buffers contained protease inhibitors PMSF (0.1 mM), TPCK (50 µm), and TLCK (50 µm).

3. Cells were washed as in protocol 1 and then lysed in 0.5% Nonidet P-40, 0.1 M NaCl, 0.25 mM sucrose, 2 mM Na-EDTA, 0.5 mM PMSF, 10 mM Na-Hepes (pH 7.5). The nuclear pellet was washed again in lysis buffer, then twice in 0.25 mM sucrose, 0.1 M NaCl, 0.5 mM CaCl_2, 1 mM Na-EDTA, 10 mM Na-Hepes (pH 7.5). Digestion with staphylococcal nuclease was carried out in this buffer. Digestion was terminated by the addition of EDTA and EGTA to final concentrations of 2 mM and 1 mM, respectively. The suspension was incubated at 4 °C for 30 min with intermittent vortexing and then centrifuged for 5 min at 12,000 X g to pellet nonsolubilized chromatin.

**Two-dimensional Electrophoretic Analysis of Oligonucleosomes**—DNP particles from staphylococcal nuclease-digested C3 chromatin were electrophoresed in the first dimension in a low ionic strength agarose gel followed by second dimension electrophoresis of DNA fragments in an SDS-agarose gel essentially as described previously (Levinger et al., 1981). The gel was stained with ethidium bromide followed by hybridization analysis (see below).

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2 R. Kellens, personal communication.
Two-dimensional Electrophoretic Analysis of Mononucleosomes—Mononucleosomal DNP particles were resolved in the first dimension by low ionic strength electrophoresis in a 6% polyacrylamide gel (Varshavsky et al., 1977; Levinger and Varshavsky, 1980; Levinger et al., 1981).

Second dimension electrophoresis of the DNA fragments was in a 9% SDS-polyacrylamide gel as described previously (Levinger et al., 1981). The gel was stained with ethidium bromide followed by hybridization analysis (see below).

In experiments in which chromatin was isolated and digested as described in protocol 4 above, solubilized mononucleosomes were fractionated in the first dimension in 5% polyacrylamide gels containing either TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM Na-EDTA, pH 8.3) or 0.1 M NaCl, 1 mM Na-EDTA, 0.5 mM Na-EGTA, 10 mM Na-Hepes (pH 7.5).

Second dimension protein runs were carried out in acetic acid-urea-polyacrylamide gels (Panyim and Chalkley, 1969) as described previously (Levinger et al., 1981).

DNA Transfer and Hybridization—DNA was denatured with NaOH and transferred from both one- and two-dimensional agarose gels to nitrocellulose filters by blotting (Southern, 1975). Low molecular weight DNA from two-dimensional polyacrylamide gels was denatured by heating the gel at 100 °C and electrophoretically transferred as described previously (Levinger et al., 1981) to DBM paper (Alwine et al., 1977). Hybridization of transferred DNA with 32P-labeled pDHFR11 (Nonberg et al., 1980) or mouse satellite DNA probes were carried out as described previously for other probes (Levinger et al., 1981). The purified mouse satellite DNA was a gift from Dr. J. Maio (Albert Einstein College of Medicine, New York, NY).

RESULTS

Characterization of Methotrexate-resistant C3 Cells—L5178Y-R (C3) is a murine lymphoblastoid cell line selected for resistance to methotrexate concentrations of up to 1 mM (Dolnick et al., 1979). Methotrexate resistance in these cells is due to a massive amplification of the gene for DHF reductase (Schimke et al., 1977). The amplified DHF reductase genes are located in one or a few contiguous chromosomal regions called "homogeneously staining regions" (Biedler and Spengler, 1976; Dolnick et al., 1979).

We determined the DHF reductase gene dosage in the C3 cells by a modified dot hybridization assay (Kafatos et al., 1979; Thomas, 1980) as described previously (Varshavsky, 1981). As can be seen from Fig. 1, D1 and D2, 0.01 μg of C3 DNA binds a 3 to 4 times greater amount of the DHF reductase hybridization probe than 1 μg of DNA from methotrexate-sensitive 3T6 mouse cells. We conclude that the DHF reductase gene is amplified 300 to 400 times in C3 cells, in good agreement with earlier estimates obtained by Cot curve analysis (Dolnick et al., 1979). In this case and elsewhere when quantitative estimates were required, we determined relative intensities by taking several different exposures of each autoradiogram and determining the difference in the time of exposure needed to produce an equal intensity in the species in question. The hybridization probe used in these and subsequent experiments, pDHFR11, hybridizes to several dis-

![Fig. 1. Comparison of dihydrofolate reductase gene, RNA, and protein dosages in methotrexate-resistant L5178Y-R(C3) cells and methotrexate-sensitive 3T6 cells. A, total cytoplasmic RNA was extracted from C3 and 3T6 cells and poly(A)* RNA was purified as described under "Experimental Procedures." Denatured RNA samples were electrophoresed in a 1.5% agarose gel and visualized by staining with ethidium bromide. Lane 1, approximately 200 μg of poly(A)* RNA from C3 cells; lane 2, approximately 20 μg of poly(A)* RNA from 3T6 cells; lane 3, HincII-digested, denatured φX174 RF DNA (a marker); lane 4, same as in lane 2 but from 3T6 cells; lane 5, same as in lane 1, but from 3T6 cells. B, fractionated RNA shown in A was transferred to DBM paper and hybridized with 32P-labeled pDHFR11. Lane 1, poly(A)* C3 RNA; lane 2, poly(A)* C3 RNA; lane 3, poly(A)* 3T6 RNA; lane 4, poly(A)* 3T6 RNA. C, purified high molecular DNA from C3 cells was digested with Bam HI, electrophoresed in an 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized to the [35P]pDHFR11. Lower autoradiographic exposures of the hybridization pattern show that the 25-kb DHF reductase-specific band is a doublet. D, relative DHF reductase gene dosage was determined by a dot hybridization technique (Kafatos et al., 1979) as described previously (Varshavsky, 1981) using pDHFR11 as a probe. D1, standard (33P)DNA spotted in a 2-fold dilution series. D2, 1 μg of the purified 3T6 DNA was spotted in the center of the nitrocellulose filter and 0.01 to 0.16 μg of the purified C3 DNA was spotted around it. E, protein fractions from equal amounts of C3 and 3T6 cells were run in an SDS-polyacrylamide gel and stained with Coomassie. Lane 1, C3 cytoplasmic fraction; lane 2, 3T6 cytoplasmic fraction; lane 3, marker channel; lane 4, C3 nuclear fraction; lane 5, 3T6 nuclear fraction. Arrow to the left of lane 1 indicates the 21.5-kilodalton band of the DHF reductase protein. Marker proteins are: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (39,000), α-lactalbumin (20,000), and soybean trypsin inhibitor (14,000) (Pharmacia).]
crete DNA species in the fractionated Bam HI digest of the purified C3 DNA (Fig. 1C), in agreement with the results of Nunberg et al. (1980).

We also compared titers of the DHF reductase mRNA in C3 and 3T6 cells (Fig. 1, A and B). Total cytoplasmic RNAs from C3 and 3T6 cells were isolated and fractionated into poly(A)⁺ and poly(A)⁻ fractions, then electrophoresed in an agarose gel (Fig. 1A), transferred to DBM paper, and hybridized to the cloned [3ZP]DHF reductase cDNA probe (Fig. 1B).

Fig. 1A and B shows that for a constant amount of the total poly(A)⁺ RNA there is an immense excess of the DHF reductase mRNA from C3 cells over that from 3T6 cells. The difference is at least 250-fold and may be even higher, suggesting that a considerable proportion, if not all, of the amplified DHF reductase genes are transcriptionally active. Not distinguished in lane 2 of Fig. 1B, but clearly seen upon lower autoradiographic exposure (not shown), are four DHF reductase poly(A)⁺ RNA species of about 1600, 1200, 1000, and 750 bases long, in agreement with the data of Setzer et al. (1980).

Polyacrylamide-SDS gel analysis of cytoplasmic and nuclear protein fractions from C3 and 3T6 cells (Fig. 1E) confirms a great overproduction of the DHF reductase protein in C3 cells (Fig. 1E1) and its cytoplasmic localization, in agreement with earlier findings (Schimke et al., 1977; Dolnick et al., 1979).

The Amplified DHF Reductase Genes Possess a Wide Range of Nucleosomal Repeat Lengths—C3 chromatin was digested with staphylococcal nuclease; solubilized oligonucleosomes were electrophoresed in the first (DNP) dimension in an 0.8% low ionic strength agarose gel followed by second dimension DNA electrophoresis (Fig. 2). The gel was stained with ethidium bromide (Fig. 2A), and the DNA was transferred to DBM paper for hybridization, at first with the DHF reductase (Fig. 2B) and then with the satellite DNA probe (Fig. 2C; see "Experimental Procedures").

The total (ethidium-stained) two-dimensional oligonucleosomal DNP-DNA pattern is virtually indistinguishable from the DHF reductase-specific hybridization pattern (compare A and B of Fig. 2). Thus, the range of nucleosomal DNA repeat lengths in the bulk chromatin is apparently close to if not identical with the range observed in the DHF reductase chromosomal domain. This finding does not by itself eliminate the possibility that nucleosomes are nonrandomly positioned along the ~32-kb-long DHF reductase gene; however, any DHF reductase nucleosome arrangement with a unique repeat length is not consistent with these data (Fig. 2B).

A very different result was obtained when the mouse satellite DNA probe was hybridized to the same total DNA pattern (Fig. 2C). Satellite oligonucleosomal DNA spots are more elongated in the first (DNP) dimension and much thinner in the second (DNA) dimension, strongly suggesting that the range of nucleosomal DNA repeat lengths in the satellite chromatin is much narrower than in either DHF reductase or bulk chromatin (Fig. 2C; cf. Fig. 2, A and B). This same result was obtained previously with rat satellite chromatin (Levinger et al., 1981) which has a DNA sequence repeat length of 370 bp (the predominating DNA repeat length in mouse satellite is 240 bp (Reiss and Biro, 1978)). Another interesting feature of the satellite hybridization pattern (Fig. 2C) is a very high relative intensity of the dinucleosomal spot; note also that the range of DNA sizes within this spot is far greater than for the rest of satellite-specific oligonucleosomal DNA spots (Fig. 2C; cf. Fig. 2, A and B).

The structural meaning of these features of the satellite-specific hybridization pattern remains unknown.

Protein Components of Electrophoretically Resolved Mononucleosomes from C3 Cells—Mononucleosomes labeled in vivo with [³H]lysine were fractionated in the first (DNP) dimension in a low ionic strength polyacrylamide gel (Fig. 3B) followed by second dimension acetic acid-urea-polyacrylamide gel electrophoresis to resolve protein components of the separated mononucleosomal particles (Fig. 3A and inset). One feature of the protein pattern (Fig. 3A) is a virtually complete absence of mononucleosomes containing ubiquitin-H2A semihistone (uH2A; previously called A24 protein), a specific covalent conjugate of histone H2A and a small protein, ubi-
TPCK was used to determine whether uH2A is lost during chromatin isolation and nuclease digestion in the presence of proteinase inhibitors PMSF (0.5 mM), TLCK, and P3, as seen whether the virtual absence of uH2A in chromatin from other cultured cells, such as HeLa (Goldknopf et al., 1977; Schlesinger et al., 1975; Wu et al., 1981) and Drosophila (Levinger and Varshavsky, 1982). The uH2A-containing core (MN1) mononucleosomes migrate slightly slower than uH2A-less MN1 mononucleosomes in the first (DNP) dimension; their characteristic "signature" in the second (protein) dimension is the presence of the uH2A spot, migrating slightly slower than histone H1 (Levinger and Varshavsky, 1980; Levinger et al., 1981). Only extreme fluorographic overexposures of the pattern in Fig. 3A revealed the presence of trace amounts of uH2A-containing core mononucleosomes in nucleic digestes of C3 chromatin (data not shown). This is in contrast to considerable amounts of uH2A-containing nucleosomes in chromatin preparations from other cultured cells, such as HeLa (Goldknopf et al., 1977; Levinger and Varshavsky, 1980), mouse L1210 cells (Wu et al., 1981), and Drosophila (Levinger and Varshavsky, 1982). The relative amount of uH2A in C3 chromatin preparations was not increased even when chromatin isolation was carried out in the presence of proteinase inhibitors PMSF (0.5 mM), TPC (50 μM), and 7LC (50 μM) and an -SH-specific reagent 5,5'-dithiobis (2-nitrobenzoic acid) (0.5 mM) (see "Experimental Procedures"; data not shown). It remains to be seen whether the virtual absence of uH2A in chromatin preparations from C3 cells reflects the in vivo situation, or whether uH2A is lost during chromatin isolation and nuclease digestion as a result of activation of a uH2A-specific protease.

In vitro (see "Discussion"). Such an activity, which specifically splits uH2A into its components, histone H2A and ubiquitin, was detected recently in liver nuclei from thioacetamide-treated rats (Andersen et al., 1981).

With the exception of the absence of uH2A spots, the overall two-dimensional DNP protein pattern (Fig. 3A) is quite similar to the one observed with HeLa mononucleosomes (Levinger et al., 1981). In particular, relative mobilities of mononucleosomes containing proteins HMG14 and HMG17 (these mononucleosomes migrate both faster and slower than the H1-containing mononucleosomes) are essentially the same as those for HeLa HMG-containing mononucleosomes (Fig. 3A; cf. Levinger et al., 1981). Besides HMG14 and -17, a new HMG-like protein species is detected in HMG-containing mononucleosomes from C3 chromatin. This protein runs slightly ahead of HMG17 in the acetic acid-urea gel and resides in the same area of the two-dimensional DNP → protein patterns as the other two HMGs. Although this rapidly migrating HMG-like protein may be a partial degradation product of either HMG14 or -17, a recent report by Goodwin et al. (1981) in which a new HMG protein with similar electrophoretic properties has been identified in calf thymus mononucleosomes, makes it plausible that we are observing the C3 counterpart of the calf HMG protein. Additional minor HMG-like protein bands are present in Fig. 3A. Their distribution in the first (DNP) dimension is similar to that for HMG14 and -17 (data not shown). These proteins may be analogous to minor HMG-like proteins observed previously in HeLa mononucleosomes (Levinger et al., 1981).

Two-dimensional Hybridization Mapping of DHF Reductase and Satellite Mononucleosomes—[3H]Thymidine-labeled C3 chromatin was prepared under low ionic strength conditions and digested with staphylococcal nuclease, and the mononucleosomes obtained were electrophoresed in the first (DNP) dimension in a low ionic strength polyacrylamide gel (Fig. 4, A1 and B1, an early and a late digest, respectively). The second dimension DNA run was then carried out (Fig. 4, A2 and B2) and the DNA was denatured in situ and electrophoretically transferred to DBM paper (see "Experimental Procedures"). Hybridization of the total two-dimensional DNP-DNA patterns to the DHF reductase probe (Fig. 4, A3 and B3) and thereafter to the satellite DNA probe (Fig. 4, A4 and B4) revealed a striking similarity of the total pattern to both of the hybridization patterns. To visualize the total transferred DNA pattern, the DBM paper was impregnated with 2,5-diphenyloxazole after first removing all hybridized 32P counts as described previously (Levinger et al., 1981; see also "Experimental Procedures"). It should be noted that the presence of [3H]DNA on the DBM paper under the conditions used did not make any contribution to the autoradiographic image of the hybridized [32P]DNA, as was verified by appropriate controls (Levinger et al., 1981). The patterns of total [3H]DNA on DBM paper (Fig. 4, A5 and B5) are also very similar to the hybridization patterns (Fig. 4, A3, A4, B3, and B4), although a few minor differences were apparent. For example, in the late digest, the "whiskers" under the MN1 spot (see Levinger and Varshavsky, 1980, for a more detailed description of whiskers) are present to different extents in the total, satellite, and DHF reductase-specific patterns (Fig. 4B). The significance, if any, of these minor differences is not known.

The overall similarity of the total and DHF reductase mononucleosomal patterns includes the region of HMG-containing mononucleosomes (Fig. 4). Although most, if not all, of the amplified DHF reductase genes are apparently transcriptionally active in C3 cells (see "Discussion"), and although HMG proteins 14 and 17 are known to interact pref-
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Fig. 4. Two-dimensional hybridization mapping of DHF reductase and satellite mononucleosomes from C3 cells. [3H]Thymidine-labeled C3 chromatin prepared and digested with staphylococcal nuclease according to protocol 1 under “Experimental Procedures,” was fractionated in the first dimension low ionic strength 6% polyacrylamide gel, followed by a second dimension DNA run in a 9% SDS-polyacrylamide gel. A and B correspond to 3-min and 20-min nuclease digests, respectively, 1, ethidium-stained first dimension DNA patterns. 2, ethidium-stained second dimension DNA patterns. 3, DNA was transferred electrophoretically to DBM paper and hybridized to the [32P]pDHFR11 probe. 4, the same DBM papers were rehybridized (see “Experimental Procedures”) with the 32P-labeled satellite DNA probe. 5, the same DBM papers were treated to remove all hybridized 32P counts (see “Experimental Procedures” and Levinger et al., 1981) and fluorographed in the presence of 2,5-diphenyloxazole to visualize the patterns of transferred 3H-labeled total DNA.

HMG-containing mononucleosomes (MN_{HMG}) migrate in two groups, one just ahead of MN2 and the other just behind it (Fig. 3), similar to HeLa HMG-mononucleosomes (Levinger et al., 1981). The MN_{HMG}’s which migrate ahead of MN2 lack H1 histone and contain 1 molecule of either HMG14 or -17 per particle (Albright et al., 1980; Sandeen et al., 1980; Mar- dian et al., 1980). More than 50% of the total DNA in the corresponding area of the first (DNP) dimension pattern corresponds to MN_{HMG} DNA as determined by comparison of relative intensities of [3H]lysine-labeled or Coomassie-stained HMG and histone H4 bands in different DNP protein patterns (Fig. 3 and data not shown; see Levinger et al., 1981, for a detailed description of the procedure as applied to HeLa mononucleosomes. Composition of MN_{HMG} mononucleosomes which migrate behind MN2 in the first (DNP) dimension (Fig. 3) is mixed, consisting of MN_{2HMG}, MN_{2HMG1}, and MN_{2HMG11} (Albright et al., 1980). Both of the MN_{HMG} DNA regions, which flank the MN2 DNA spot, are represented equally in the total, DHF reductase-specific, and satellite-specific patterns with relation to “non-HMG” DNA markers.
FIG. 5. Two-dimensional hybridization mapping of DHF reductase mononucleosomes produced and fractionated under high salt conditions. C3 chromatin was prepared and digested according to protocol 4 under “Experimental Procedures.” Mononucleosomes from the same intermediate level digest were fractionated in the first dimension in 5% polyacrylamide gels with either TBE buffer (A) or 0.1 M NaCl-containing buffer (B) (see “Experimental Procedures” for details). 1, ethidium-stained first dimension DNP patterns. 2, ethidium-stained second dimension DNA patterns. 3, DNA was transferred electrophoretically to DBM paper and hybridized to the [32P]pDHFR11 probe (see “Experimental Procedures”).

MN1 and MN2 (Figs. 4 and 5).

To check whether this finding depended on particular conditions of chromatin isolation, digestion, and fractionation, we used different protocols for all three (see “Experimental Procedures”). The procedures included low ionic strength conditions throughout (Fig. 4), similar protocols but in the presence of divalent cations (data not shown), use of several different proteinase inhibitors and of an — SH-specific reagent, DTNB (see “Experimental Procedures”; data not shown), and lastly, “physiological” concentrations of monovalent cations throughout chromatin isolation, digestion, and fractionation (Fig. 5). The latter methods (see “Experimental Procedures”) were used following the results of Sandeen et al. (1980), who found that HMG-nucleosome reconstitution and electrophoresis at higher ionic strengths promotes cooperativity of HMG binding, favoring the MNhMG product. In all cases, a great similarity between the total and DHF reductase-specific hybridization patterns was observed (Figs. 4 and 5), including the relative proportions of HMG-mononucleosomal DNA in the DHF reductase-specific versus bulk chromatin regions.

The data of Fig. 5 illustrate a strong dependence of the mononucleosomal patterns on the ionic strength of the first (DNP) dimension electrophoretic system. In our 0.1 M NaCl-based mononucleosome preparation, no well defined, discrete MN2 particles are seen in either the TBE or 0.1 M NaCl gel system (Fig. 5). However, a large proportion of slowly migrating material containing longer than 146-bp DNA fragments, is seen when a relatively low ionic strength first dimension TBE electrophoretic buffer is used (conductivity of TBE is ~1/4 that of 0.1 M NaCl) (Fig. 5A). Since HMG-less, H1-containing MN2 particles are insoluble in 0.1 M NaCl (Albright et al., 1980; McGhee and Felsenfeld, 1980), the slowly migrating material in Fig. 5A probably consists of MnhMG and/or of MNhMG. In a 0.1 M NaCl-containing gel, these particles almost co-migrate with MN1, apparently as a result of losing their H1 and/or HMG components during the electrophoresis. The analogous phenomenon of electrophoresis-induced stripping of H1 histone from MN2 mononucleosomes at ionic strengths exceeding ~0.03 was observed by Albanese and Weintraub (1980).

As already mentioned in the preceding section, isolated C3 chromatin and mononucleosomes were virtually completely devoid of uH2A semihistone, in contrast to the situation with many other cell lines from different species. Our recent studies with Drosophila chromatin strongly implicated uH2A as a specific component of transcribed chromosomal fibers (Levine and Varshavsky, 1982). Since it is still possible that uH2A is present in C3 cells in vivo but is cleaved during...
chromatin isolation in vitro, further studies on C3 mononucleosomes would have to rely on some method for inhibiting uH2A-specific isopeptidase activity.

**DISCUSSION**

**Absence of Preferential Binding of HMG14 and -17 to DHF Reductase Genes in Isolated C3 Chromatin: Possible Interpretations**—The major result of this work is the conclusion that although the amplified DHF reductase genes are apparently transcribed in C3 cells, there is no preferential association of HMGs with the DHF reductase mononucleosomes in chromatin isolated from unsynchronized C3 cells. Minor differences in HMG content might be missed by our procedure, but it is clear that there are no large differences in HMG content between transcribed DHF reductase and nontranscribed satellite DNA sequences in isolated mononucleosomes (see “Results”). Preferential association of HMGs with DHF reductase mononucleosomes was expected by analogy with results from other laboratories obtained with a very different system, chicken erythroid cells (Weisbrod et al., 1980; Sandeen et al., 1980). According to those data, HMG14 and -17 bind preferentially to globin DNA-containing nucleosomes isolated from terminally differentiating chicken erythroblasts. Our results clearly do not contradict, at least at the present level of understanding, the concept of preferential HMG binding to some active genes; several different interpretations are formally compatible with our data. Below we enumerate and briefly discuss some of these interpretations.

1. The assumption that a considerable proportion of the amplified DHF reductase genes are active in C3 cells may be invalid; if only a small fraction of about 350 DHF reductase gene copies per C3 cell is transcriptionally active, the preferential association of HMGs with these few genes might not be seen over a background of nonspecific HMG interactions with the bulk of inactive DHF reductase genes.

Since no direct estimate of the proportion of transcribed DHF reductase genes in an unsynchronized population of C3 cells is available, this interpretation cannot be refuted at the present time. It is unlikely, however, that only a few out of approximately 350 DHF reductase genes are actually transcribed, since first, the degree of DHF reductase gene amplification is a strong function of the selecting methotrexate concentration (Selumke et al., 1977; Varshavsky, 1981), suggesting that all or almost all amplified DHF reductase genes participate in the production of the DHF reductase mRNA. Second, it is unlikely that at least a 250-fold overproduction of the DHF reductase mRNA by C3 cells (see “Results”) is due to a very high activity of a small fraction of the amplified DHF reductase genes, in the absence of any activity of the rest of them.

2. It is possible that HMGs do interact preferentially with the DHF reductase genes in vitro but redistribute between different nucleosomes during chromatin isolation, digestion, and/or fractionation, so that the in vitro HMG distribution is no longer DHF reductase-specific.

Although this interpretation cannot be refuted at the present time, and moreover, nucleosome-bound HMG proteins are known to redistribute in vitro under certain solvent conditions (Albright et al., 1980), we showed that a wide variety of chromatin isolation, digestion, and fractionation protocols (see “Experimental Procedures” and “Results”) all produce the same apparently nonspecific in vitro HMG distribution with regard to the DHF reductase genes. Some of these protocols were identical with those used with the globin gene/chicken erythroid cell system in which preferential HMG binding to nucleosomes containing globin-specific DNA sequences was convincingly demonstrated (Weisbrod and Weintraub, 1981; Sandeen et al., 1980).

3. Current estimates of the level of transcriptional activity of the “housekeeping” DHF reductase gene in noncycling, methotrexate-sensitive cells place it at 10- to 100-fold lower than the level of activity of differentiation-specific “luxury” genes such as those for globin and ovalbumin in terminally differentiating chicken erythroid and oviduct cells (Mathis et al., 1980). It now appears that the DHF reductase gene is transcribed at an approximately constant rate throughout the cell cycle. We have previously suggested that maintenance of preferential associations of HMGs with a transcribed chromatin fiber may be dependent upon frequent passages of transcribing RNA polymerases along the fiber (Levinger et al., 1981). In other words, it is possible that the linear density of HMG binding to nucleosomes along a given chromatin fiber in vitro is a function of the frequency of transcription of that fiber. If so, highly active genes, such as the globin gene in erythrocyts, would be expected to have more HMGs bound per nucleosome than the relatively slowly transcribed genes, such as DHF reductase.

**Ubiquitin-H2A Semihistone Is Virtually Absent from Isolated C3 Chromatin**—One unusual feature of C3 chromatin and mononucleosome preparations was the almost complete absence of uH2A, a specific covalent conjugate of histone H2A with a small protein, ubiquitin (see Fig. 4). uH2A semihistone substitutes for about 5 to 15% of nucleosomal histone H2A in chromatin from a wide variety of animal tissues and established cell lines, from man to Drosophila (Goldknopf et al., 1977; Kleinschmidt and Martinson, 1981; Levinger and Varshavsky, 1982). The unique structure of uH2A (Goldknopf et al., 1977), its unusual metabolic properties (Wu et al., 1981), and universal occurrence coupled with the recently discovered participation of free ubiquitin in the functioning of the cytoplasmic ATP-dependent proteolytic system (Rershko et al., 1980; Wilkinson et al., 1980) all suggest that uH2A serves some important chromosomal function.

Application of the technique for two-dimensional hybridization mapping of nucleosomes to Drosophila chromatin has led us recently to the discovery that uH2A-containing Drosophila mononucleosomes are derived specifically from transcribed chromosomal fibers (Levinger and Varshavsky, 1982). Therefore, it would be important to know whether the virtual absence of uH2A from isolated C3 chromatin (see “Results”) is due to its artificial degradation by a uH2A-specific isopeptidase (Andersen et al., 1981) during chromatin and mononucleosome preparation in vitro or whether the lack of uH2A in cells with strongly amplified specific genes may have some physiological significance.

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Chromatin Structure of DHF Reductase Gene


On the chromatin structure of the amplified, transcriptionally active gene for dihydrofolate reductase in mouse cells.
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