Enrichment of Selected Active Human Gene Sequences in the Placental Deoxyribonucleic Acid Fraction Associated with Tightly Bound Nonhistone Chromosomal Proteins†

Gary L. Norman and Isaac Bekhor*

ABSTRACT: A DNA fraction which is highly enriched in active gene sequences and tightly associated with a subset of nonhistone chromosomal proteins has been isolated from human placenta. After extraction with 2 M NaCl, placental chromatin was separated into two distinct components by centrifugation. Of the total DNA, ~96% (DNA-S) is protein free, while the remaining 4% (DNA-P) is tightly complexed with nonhistone chromosomal proteins. Reassociation studies revealed that the DNA-P fraction was enriched 22-fold in actively transcribed human placental lactogen gene sequences, while the DNA-S fraction was correspondingly depleted 22-fold in these sequences. Approximately 45% of the sequences present in DNA-P (equivalent to 1.8% of the genome) were not present in the DNA-S fraction. Reassociation of nick-translated DNA-P to DNA from a partial digest of DNase I treated nuclei indicated that 27% of the DNA-P sequences were DNase I sensitive, suggesting they may represent actively transcribed gene sequences. Analysis of the overall sequence organization of DNA-P showed that relative to unfractionated DNA and DNA-S, DNA-P was enriched in single-copy sequences, slightly enriched in the class of middle repetitive sequences from C_0t 0.01 to 100 M·s, devoid of the more highly repetitive sequences ($C_0t \leq 0.01$). The distribution of total active placental genes between DNA-P and DNA-S was measured by hybridization with a complementary DNA probe transcribed from total polysomal poly(A+) messenger RNA. We found that 57% of this cDNA probe reassociated to DNA-P and 58% to DNA-S, while 95% reassociated to DNA-P mixed with DNA-S at the observed ratio of 4 to 96, suggesting that the DNA-P fraction contained a different population of active gene sequences than DNA-S. From these results we estimate that ~85% of the transcribed sequences appear to be distinctly distributed and equally proportioned between DNA-P and DNA-S, while ~15% of the transcribed sequences are common to both fractions. We suggest that the strong affinity of the tightly bound nonhistone chromosomal proteins for the DNA-P fraction indicates a likely role for these proteins in the regulation of gene expression.

Of the two groups of nuclear proteins, the histones and the nonhistone chromosomal proteins (NHCP),[1] it has been possible to characterize the histones primarily due to their relatively small number and large quantity. The four histones H2a, H2b, H3, and H4 organize the DNA into nucleosomes (McGhee & Felsenfeld, 1980), and although the basic nucleosome structure does not appear sufficient to confer transcriptional regulation, particular nucleosomes may modify transcription by acetylation and phosphorylation of histones (Louie & Dixon, 1972; Ruiz-Carrillo et al., 1975), by phasing of the histone core relative to the DNA nucleotide sequences (Ponder & Crawford, 1977; Wittig & Wittig, 1979), and by interactions with NHCP's (Liew & Chan, 1976; Defer et al., 1978) including the HMG (high mobility group) proteins (Goodwin et al., 1979). Classes of nonhistones showing higher affinity for DNA have been implicated in specific regulation of transcription (Chiu et al., 1975). The difficulty in studying the significance of NHCP has been the identification of a specific protein or group of proteins from the multitude of chromosomal proteins in the nucleus which may be important in transcriptional control.

It has been suggested that multiple sequence-specific contacts with the DNA are necessary for the preferential interaction of a protein with a specific regulatory control sequence (Pribnow, 1979). The binding affinities to control sequences must be several orders of magnitude higher than the binding to the nonspecific sequences (Pribnow, 1979) to ensure the preferential binding of proteins to control sequences rather than to nonspecific (nonregulatory) sequences. We previously estimated that $K_{binding}$ for histones in chromatin falls in the range of 10^8 M^{-1}, while $K_{binding}$ for the nonhistones ranges from 10^9 to 10^{12} M^{-1}, with the most tightly bound proteins exhibiting a $K_{binding}$ greater than 10^{12} M^{-1} (Lapeyre & Bekhor, 1976). Thus the tightly bound chromosomal proteins, which constitute ~5% of the total NHCP, are a likely group in which to search for transcriptionally important molecules. Our laboratory has made a functional separation of the NHCP based on protein–DNA interaction strengths and has reported that the tightly bound NHCP group (proteins resistant to extraction from chromatin with 5 M urea–1 M NaCl) can significantly enhance transcription of DNA complexed with histones with a concomitant enhancement in the amount of bound homologous RNA polymerase II (Bekhor & Samal, 1977). In addition, the tightly bound chromosomal proteins have been shown to bind androgen–receptor complexes (Wang, 1978), to contain specific "acceptor" protein molecules for progesterone–receptor complexes (Spelsberg et al., 1976), to include

† From the Laboratory for Molecular Genetics and Department of Basic Sciences, School of Dentistry, University of Southern California, Los Angeles, California 90007. *Received November 14, 1980.* This work was supported by Grant DE-04031-06 from the National Institute of Dental Research, National Institutes of Health.

* Correspondence should be addressed to this author at the Laboratory for Molecular Genetics, University of Southern California, Los Angeles, CA 90007.

[1] Abbreviations used: NHCP, nonhistone chromosomal protein; HMG, high mobility group protein; DNA-T, unfractionated genomic DNA; DNA-P, chromosomal protein-bound DNA obtained from 2 M NaCl–TPD extracted chromatin; DNA-S, soluble and protein-free DNA obtained from 2 M NaCl–TPD extracted chromatin; hPL, human placental lactogen; kbp, kilobase pair; RNP, ribonucleoprotein; cDNA, complementary DNA; TPD, 10 mM Tris-HCl, 0.2 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride, pH 8.0; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid.

0006-2960/81/0420-3568$01.25/0

tissue-specific antigens (Chytil & Spelsberg, 1971), and to be essential for in vitro transcription of the chicken globin (Chiu et al., 1975) and ovalbumin genes (Tsai et al., 1976). Other evidence pointing to the particular importance of this group of proteins has come from studies on the residual protein skeleton obtained by extracting nuclei with high salts. These proteins are known as scaffolding proteins in metaphase chromosomes (Adolph et al., 1977) or nuclear matrix in interphase nuclei (Berezney & Coffey, 1977). The nuclear matrix seems to contain a high concentration of hnRNP particles and small nuclear RNA's (Miller et al., 1978a,b), HeLa cell specific antigens (Campbell et al., 1979), and specific binding sites for estradiol (Barrack & Coffey, 1980).

In view of the apparent significance of this small fraction of nuclear proteins, we recently investigated the possibility that the 2 M NaCl insoluble chromosomal proteins may in part be associated with particular DNA sequences in chicken reticulocytes. We found that a striking fractionation of DNA sequences occurs when chromatin is extracted with 2 M NaCl and the protein-bound DNA (DNA-P) is separated from protein-free DNA (DNA-S). Specifically, the tightly bound proteins are associated with ~5% or less of the chromosomal DNA. This DNA (DNA-P) was found to be enriched 20-fold in active globin gene sequences (Bekhor & Mirell, 1979). Furthermore, when a similar fractionation was done on chromatin from chicken liver, where the globin gene is inactive, the globin gene was found in the protein-free DNA-S fraction. DNA-P from liver, however, while not containing the globin gene, was shown to contain ~25% of the total active liver gene sequences (Gates & Bekhor, 1980). These dramatic results suggest that the tightly bound NHCP are associated with active genes and that this association varies directly with the transcriptional activity of specific genes.

In the present work, we have completed an extensive study of the DNA sequences found associated with the tightly bound nonhistones in the human placenta. We have determined the distribution of human placental lactogen gene (hPL; human chorionic sommatomammotropin) and total active gene sequences in both DNA-P and DNA-S and have examined the sequence organization of DNA-P relative to that of total unfractionated DNA and DNA-S. The data suggest that the tightly bound nonhistones are nonrandomly associated with DNA-P and that this DNA contains ~40% of the total active genes found in human placenta.

Experimental Procedure

Isolation of Total Poly(A+) mRNA and Lactogen mRNA from Human Placenta. Human placental lactogen poly(A+) mRNA and total poly(A^+)-mRNA were prepared and characterized in collaboration with Drs. Fehn, Lessard, and Gusseck of Loma Linda University (Loma Linda, CA). Term human placentas obtained by caesarean section were cooled on ice immediately after delivery and processed within 30 min. A 20% homogenate was prepared from 40 g of placental tissue in homogenization buffer (7 M guanidinium thiocyanate, 0.1 M β-mercaptoethanol, 50 mM EDTA, and 50 mM Tris-HCl, pH 7.4) by using a Brinckmann Polytron. Cellular debris was removed by centrifugation at 10 000 rpm for 10 min and to the supernatant 0.2 g of optically pure CsCl was added per mL, heated to 55 °C, and then centrifuged at 15 °C for 16 h at 25 000 rpm in a SW-27 rotor to sediment RNA (Rowe et al., 1978). The RNA pellets were dissolved in doubly distilled water and extracted with chloroform–butanol (4:1) and then made 0.1 M in NaCl, and the RNA was precipitated at −20 °C by the addition of 2.5 volumes of ethanol. About 0.5 mg of RNA was obtained from 1 g of tissue. Total poly(A+) mRNA was isolated from total cellular RNA by the repeat binding procedure of Bantle et al. (1976) using oligo(dT)–cellulose (Collaborative Research, T-3). Twice bound RNA was stored at −20 °C in sterile water. The mRNA comprised ~1% of the total cellular RNA. Isolated poly(A+) mRNA was further fractionated on 1.3% agarose (10 cm) tube gels containing CH_3HgOH (Bailey & Davidson, 1976) and sized with appropriate RNA markers. RNA was initially diluted with an equal volume of sample buffer (50 mM H_3BO_3, 5 mM $Na_2B_4O_7$, 10 mM Na_2SO_4, 1 mM EDTA, 10 mM CH_3HgOH, and 10% glycerol, pH 8.2), and electrophoresis was carried out at 5 mA/tube for 1.5 h at room temperature. RNA mobilities were determined by scanning the gels at 260 nm. Selected RNA bands were excised from the gel and transferred to the micropreparative electrophoresis system essentially as described by Case and Daneholt (1976). Following electrophoresis, the eluate was mixed with 0.1 volume of 0.1 M dithiothreitol and incubated for 30 min at room temperature, and the RNA precipitated with ethanol. In vitro translation of ~1–2 μg of specific RNA's was performed according to the method of Pelham & Jackson (1976) using [^{35}S]methionine (437 Ci/mmol; New England Nuclear) as the tracer in a rabbit reticulocyte lysate translation system (Allen & Schweet, 1962), in which endogenous RNA was removed by treatment with micrococcal nuclease. The nuclease was inactivated by the addition of ethylene glycol bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) to 0.1 M (Pelham & Jackson, 1976). Translation products from total poly(A+) mRNA were analyzed on sodium dodecyl sulfate–acrylamide gels (Laemmli, 1970) which were impregnated with PPO (diphenylozaxole) for localization of labeled polypeptides by fluorography (Bonner & Laskey, 1974). Specific human placental prolactogen (or lactogen) synthesis was assayed for by immunoprecipitation (Martial et al., 1977) with sheep antiserum generated against purified hPL (Handwerger & Sherwood, 1974). Formalin treated Cowan I strain, *Staphylococcus aureus* (Pansorbin, Calbiochem-Behring), replaced the second antibody in the precipitation and sedimentation of antibody–antigen complexes (Kessler, 1975). Pelleted antibody–hPL complexes were solubilized by the addition of sodium dodecyl sulfate ($NaDodSO_4$) to 2% and heating for 3 min in a boiling water bath. Samples were later analyzed on 12.5% polyacrylamide–$NaDodSO_4$ gels (Laemmli, 1970), and the labeled polypeptides were localized by fluorography (Bonner & Laskey, 1974). The poly(A+) mRNA coding for prolactogen, which migrated as a 12–13S band on CH_3HgOH–1.3% agarose gels, constituted ~5–10% of the total poly(A+) mRNA.

Isolation of Nuclei. All steps were performed at 4 °C unless otherwise noted. The soft placental tissue was dissected from the placenta, rinsed with saline, and weighed. The tissue (200–400 g) was added to 1 L of grinding buffer (0.25 M sucrose, 1 mM $MgCl_2$, 0.1 M PMSF, and 50 mM Tris-HCl, pH 7.5) and mixed for 5-s bursts in a blender to disperse the cells, and the suspension was centrifuged at 4000 rpm for 10 min in a Sorvall GSA rotor to pellet the cells. The pellet was suspended into sterile distilled water to lyse red blood cells, and the suspension immediately centrifuged at 4000 rpm for 10 min. The resulting cellular pellet was suspended in nuclear buffer I (0.1 M NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0) and recollected by centrifugation at 6000 rpm for 10 min. This step was repeated as necessary to remove any residual blood cells in the pellet. The washed pellet was resuspended in nuclear buffer II (0.1 M NaCl, 1.5 mM $MgCl_2$, 0.1 mM PMSF, 0.2 mM DTT, 10 mM Tris-HCl, pH 8.0, and

0.2% Triton X-100) to lyse the cells, and crude nuclei were collected by centrifugation at 10000 rpm for 10 min. The nuclei were freed of Triton X-100 by repeated suspension in nuclear buffer III (0.1 M NaCl, 1.5 mM $MgCl_2$, 0.1 mM PMSF, 0.2 mM DTT, and 10 mM Tris-HCl, pH 8.0) and centrifugation at 10000 rpm for 10 min. Nuclei were further purified by suspension in 5 volumes of 2.2 M sucrose in nuclear buffer III, layering over 5 mL of 2.2 M sucrose in nuclear buffer III, and centriguation at 25000 rpm for 60 min in a SW-27 rotor to pellet purified nuclei.

Isolation of Placental Chromatin. Purified nuclei were suspended in TPD (10 mM Tris-HCl, pH 8.0, 1.0 mM PMSF, and 0.2 mM DTT), allowed to swell for 20 min, and lysed by homogenization. Nuclear lysis was confirmed by phase-contrast microscopy. This crude chromatin suspension (10-mL portions) was layered over 25 mL of 1.1 M sucrose in TPD and centrifuged at 2500 rpm for 15 min in a Beckman SW-27 rotor to pellet nucleoli. The partially purified chromatin over the interphase was removed and layered over 20 mL of 1.7 M sucrose in TPD, stirred lightly to break the interphase, and centrifuged at 26000 rpm in a SW-27 rotor for 90 min to pellet purified chromatin.

Separation of Chromatin into Protein-Free (DNA-S) and Protein-Bound DNA (DNA-P). Fractionation of chromatin into DNA-S and DNA-P was performed essentially as previously described (Bekhor & Mirell, 1979). Purified chromatin was suspended in 2 M NaCl–TPD at an A_{260} of 5 and extracted for 30 min. The viscous suspension (in 25-mL portions) was layered over a 10-mL cushion of 1 M sucrose–2 M NaCl in TPD and centrifuged for 20 h at 60000 rpm in a Beckman-type 60Ti rotor. The resulting pellet consisted of a brownish DNA–protein complex (DNA-P) overlaid by a clear protein-free DNA (DNA-S). The entire DNA pellet, including both DNA-P and DNA-S, was suspended in TPD at an A_{260} of 20 and dialyzed overnight against 4 L of TPD to aggregate DNA-P. The dialyzed DNA was centrifuged at 10000 rpm for 10 min in a Sorvall SS-34 rotor to pellet the DNA-P. The soluble (protein-free) DNA-S remained in the supernatant. DNA-P was freed of contaminating DNA-S by repeated washings (5–6 times) with TPD. Spectrophotometric monitoring of the DNA-P pellet and the TPD washes indicated the progressive decontamination of the DNA-P with respect to both the characteristic DNA–protein profile obtained from DNA-P and the decrease in the A_{260} of the TPD washes toward zero. The yield in DNA-P was 3–6% of the total nuclear DNA (DNA-T).

Nuclease Digestion of Placental Nuclei. Purified placental nuclei were suspended into DNase I buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 3 mM $MgCl_2$) at a DNA concentration of 1 mg/mL and digested with 20 μg/mL of electrophoretically purified DNase I (Worthington) at 37 °C until 15% of the nuclear DNA was rendered acid soluble (Weintraub & Groudine, 1976). Reactions were terminated by adding EDTA to 5 mM. For micrococcal nuclease digestion of nuclei, nuclei were suspended into DNase I buffer which had been made 0.05 mM in $CaCl_2$ and digested at 37 °C with 10 units of enzyme/100 μg of DNA until ~35–40% of nuclear DNA was rendered acid soluble. Digestion was stopped by adding EDTA and $NaDodSO_4$ to 5 mM and 1%, respectively. The sizes of the DNA's resistant to digestion with either DNase I or micrococcal nuclease were analyzed on 1 and 3% agarose gels as described below. DNase I or micrococcal nuclease treatment of nuclei under the conditions described generally resulted in DNA's of an average base-pair length of 2000 or 1000, respectively.

Purification of DNA. All DNA-containing samples were suspended in 10 mM Tris-HCl, pH 8.0, containing 1% sodium dodecyl sulfate, and incubated with 50 μg/mL proteinase K (Boehringer-Mannheim) for 2 h at 37 °C. The solution was extracted twice for 20 min with chloroform–isopentyl alcohol (24:1), and the mixture centrifuged to resolve the phases. The aqueous phase was made 0.24 M in ammonium acetate, 2.5 volumes of cold ethanol added, and the DNA precipitated overnight at −20 °C. The precipitated DNA was dissolved in 10 mM Tris-HCl, pH 8.0, 20 μg/mL RNase A (previously boiled for 10 min to inactivate any contaminating DNase) added, and the solution incubated at 37 °C for 2 h. Proteinase K (50 μg/mL) and $NaDodSO_4$ to 1% were added and the incubation was continued for an additional 2 h. The solution was then extracted twice with chloroform–isopentyl alcohol (24:1) as above and once with chloroform–isopentyl alcohol–phenol (24:1:25). The final aqueous phase was made 0.24 M in ammonium acetate and precipitated with 2.5 volumes of ethanol. The precipitated DNA was dissolved in distilled water and dialyzed against 4 L of 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 0.3 M NaCl. DNA judged to be spectrally pure (A_{260}/A_{230} = 2.4; A_{260}/A_{280} = 1.8–1.9) was stored at −90 °C. DNA to be used for reassociation studies was further purified by first shearing the DNA to ~500 bp as described below and then heating the sheared product in 0.3 M NaOH for 15 min at 70 °C to ensure hydrolysis of any residual RNA. The denaturated DNA was cooled, neutralized with HCl, and stored −90 °C until use.

Synthesis of [^{3}H]cDNA. The synthesis of [^{3}H]cDNA to hPL mRNA and total polysomal poly(A+) mRNA was performed essentially as described by Friedman & Rosbash (1977). To a final reaction volume of 25 μL containing 80 μM [^{3}H]dCTP (24 Ci/mmol; New England Nuclear), 50 mM Tris-HCl, pH 8.3, 60 mM NaCl, 20 mM DTT, 100 μg/mL actinomycin D, 8 μg/mL oligo(dT), 1 mM each of dATP, dGTP, and TTP, and 0.5 μg of either hPL mRNA or total poly(A+) mRNA was added with 400 units/mL avian myeloblastosis virus reverse transcriptase (a gift from Dr. J. Beard, Life Sciences, Inc.). The reaction mixture was incubated for 30 min at 37 °C. Synthesis was stopped by the addition of EDTA and $NaDodSO_4$ to final concentrations of 10 mM and 1%, respectively. This solution was made 0.3 M in NaOH, incubated at 70 °C for 15 min to hydrolyze the RNA template, and cooled on ice, 0.25 μL of phenol red was added, and the solution was neutralized with 1 N HCl. After addition of yeast tRNA (50 μg), the cDNA solution was loaded on a siliconized sterile Sephadex G-50 column (50 × 0.8 cm) which had been equilibrated with the running buffer (20 mM Tris-HCl, pH 7.5, 65 mM NaCl, and 10 mM EDTA). Fractions of 0.5 mL were collected in sterile plastic tubes and 10-μL aliquots of each fraction were counted in toluene–Triton X-100 (2:1) scintillation cocktail. The fractions containing [^{3}H]cDNA were pooled, ammonium acetate was added to a final concentration of 0.24 M, and the cDNA was precipitated with 2.5 volumes of ethanol at −20 °C. The precipitated cDNA was dissolved in sterile distilled water at a concentration of 10^6 cpm/mL and frozen at −90 °C until use. Specific activity of the purified cDNA was 2×10^7 cpm/μg. The cDNA's transcribed from hPL mRNA or from total poly(A+) mRNA were found to be from 500–900 bases long as assayed by fluorography.

Nick Translation of DNA. Purified DNA (DNA-P, DNA-S, and total unfractionated DNA) was nick translated by using a modification of the method of Balmain & Birnie (1979). To a buffer solution containing 20 mM Tris-HCl, pH

7.9, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 50 μg/mL bovine serum albumin, 15 μM each of dATP, dGTP, and TTP, 15 μM [^{3}H]dCTP (24 Ci/mmol; New England Nuclear), and 0.5 ng of electrophoretically purified DNase I/μg of DNA, 1–2 μg of purified DNA and 5 units of *Escherichia coli* DNA polymerase I (Boehringer-Mannheim) per μg of DNA were added (final volume = 100 μL). The mixture was incubated at 14 °C for 2 h and the reaction stopped by adding EDTA to 20 mM. Fifty micrograms of yeast tRNA was added to the solution as the carrier, and following extraction with an equal volume of phenol, the nick-translated product was separated from unincorporated nucleotides by passage through a Sephadex G-50 column (50 × 0.8 cm) previously equilibrated with 0.1 SSC (SSC = 0.15 M NaCl and 0.01 M sodium citrate) running buffer. Peak fractions were identified by spotting 10-μL aliquots onto DE-81 Whatman papers which were washed and counted as described below under Reassociation. Specific activities of nick-translated DNA's ranged from 1×10^6 to 2×10^6 cpm/μg of DNA.

Shearing and Sizing of DNA. DNA for reassociation experiments was sheared to a length of ~400–500 bp by using a modification of the procedure of Davis & Phillips (1978). Purified DNA samples dissolved at A_{260} of 5 in 10 mM Tris-HCl, pH 8.0, and 1 M NaCl were cooled in a NaCl–ice-water bath for 30 min. DNA was sonicated in 5-s bursts with 2-min intervals at 350 W with a Braunsonic 1510 sonicator for a total of 3 min. Prior to sonication the intermediate probe was precooled, and during the intervals between sonication the probe was cooled by the application of dry ice. Sheared DNA was precipitated with 2.5 volumes of cold ethanol at −20 °C. The precipitated sheared DNA was dissolved in 10 mM Tris-HCl, pH 8.0, and passed through a Chelex-100 (Bio-Rad) column (5 × 0.5 cm). The size of the DNA fragments was determined by electrophoresis on a 1 or 3% agarose gel, using the Tris–acetate (50 mM Tris-HCl, 20 mM sodium acetate, and 2 mM EDTA, pH 8.3) system of Helling et al. (1974). DNA samples were mixed with 25 μL of running buffer containing 0.5 μL of 1% bromophenol blue as the tracking dye, and electrophoresis was carried out for 6.5 h at 50 V. Phage λ DNA cleaved with *Eco*RI and φχ 174RF DNA cleaved with *Hae*III restriction enzymes were used as markers. Following electrophoresis, the gels were stained with 0.5 μg/mL ethidium bromide and the DNA was visualized by ultraviolet illumination. The size of cDNA and nick-translated DNA samples was determined by subjecting the gels to fluorography as described by Laskey & Mills (1975). Since we found nick-translated DNA's to be of approximately the same size as the substrate DNA (>20 kbp), the labeled DNA's were sheared as described to an approximate size of 400–500 bp prior to reassociation studies. DNA from DNase I and micrococcal nuclease treated nuclei were also sheared to average base lengths of 400–500 bp. A component of 200 bp monomer was present in DNA's from micrococcal nuclease treated nuclei.

Reassociation. Reassociation of [^{3}H]cDNA and ^{3}H-labeled nick-translated DNA probes to various driver DNA's was carried out at 68 °C in 0.3 M NaCl, 50 mM Tris-HCl, pH 7.9, and 0.1% $NaDodSO_4$, as described by Weintraub & Groudine (1976). For each C_0t (moles of nucleotides per liter × seconds) point three individual 0.6-mL polypropylene tubes were prepared for triplicate runs. Each tube contained the appropriate amount of driver DNA to reach the desired C_0t points (from 0.01 to ~45 000 M·s) in 96–144 h of incubation and 2000 cpm of [^{3}H]cDNA or ^{3}H-labeled nick-translated DNA's in a final volume of 10 μL. The reaction mixtures were overlaid with ~50 μL of mineral oil, tubes were capped, and the DNA was denatured by heating in a 95 °C water bath for 30 min. All tubes, with the exception of zero-time samples used to measure the S1 nuclease resistance of the probe, were immersed in a 68 °C water bath and incubated for 96 h (for C_0t from 0.01 to 10 000 M·s) or 144 h (for C_0t points over 10 000 M·s). Reassociation was stopped by immersing the tubes in an ice-water bath, adding 40 μL of S1 nuclease digestion buffer (30 mM sodium acetate, pH 4.5, 0.15 M NaCl, and 1 mM $ZnSO_4$), and freezing the tubes at −20 °C until the time of S1 digestion assay. The extent of DNA reassociation was determined by digesting unannealed DNA with 1000 units of S1 nuclease (Calbiochem-Behring) for 40 min at 40 °C. The amount of nuclease resistant DNA hybrids was assayed by applying the contents of each tube to individual DE-81 ion-exchange paper (Whatman) and washing the papers 6 times with 0.5 M dibasic sodium phosphate, 2 times with deionized water, 2 times with ethanol, and once with acetone. Filters were air-dried and then counted in a toluene–PPO scintillation cocktail [14 g of 2a70 (Research Products, Inc.), 14 mL of H_2O, 70 mL of NCS (Amersham), and 3.5 L of toluene].

Under the hybridization conditions described above (incubation for a constant time period under 50 μL of mineral oil), we observed no change in NaCl concentrations in the aqueous phase. This was determined by conductivity measurements of the aqueous phase prior to, during, and following incubation at 68 °C for 144 h. Incubation periods of 144 h resulted in a loss of ~7% in trichloroacetic acid precipitable counts, suggesting that the DNA probes became subject to degradation during long periods of incubation at 68 °C. Self-annealing of nick-translated DNA's was also monitored by incubation of the probes with sheared heterologous *E. coli* DNA at driver concentrations equivalent to those used for homologous driver DNA's. Under these conditions we did not find significant self-annealing reactions to occur with our DNA's. S1 nuclease resistant counts for self-annealed DNA's (if any) were either equivalent to or below our observed zero-time resistance. Zero-time resistant counts were at levels below 5% of input cpm.

Other Methods. Polyacrylamide gel electrophoresis of chromosomal proteins was carried out on slab gels in buffers as described by Laemmli (1970). Concentrations of DNA were determined by the method of Burton (1955), RNA by the method of Dische & Schwarz (1937), and protein by the methods of Hartree (1972) or Bradford (1976).

Results

Human Placental Lactogen Messenger RNA. The hPL mRNA used as template for the synthesis of hPL cDNA was isolated from term placental tissue by chromatography on oligo(dT)–cellulose and subsequently purified by electrophoresis on 1.3% agarose gels containing CH_3HgOH. The resulting RNA, specific for pro-hPL, migrated essentially as one band at ~12–13 S (2.9×10^5 daltons; Figure 1). A complexity of 8.31×10^2 was calculated for the 2.9×10^5 dalton mRNA as determined from hybridization of pro-hPL cDNA with its template pro-hPL mRNA ($R_0t_{1/2} = 8.6 \times 10^{-4}$ M·s; R. Fehn, D. Gallo, and G. M. Lessard, personal communication, 1980). The calculated complexity (831 nucleotides) compares favorably with the length of prolactogen mRNA (916 nucleotides) determined from the molecular weight analysis of the 12–13S mRNA purified by the methods described above. In the rabbit reticulocyte cell-free translation system, this RNA directed the synthesis of only one major 24 500-dalton protein, corresponding to pro-hPL (Figure 1,

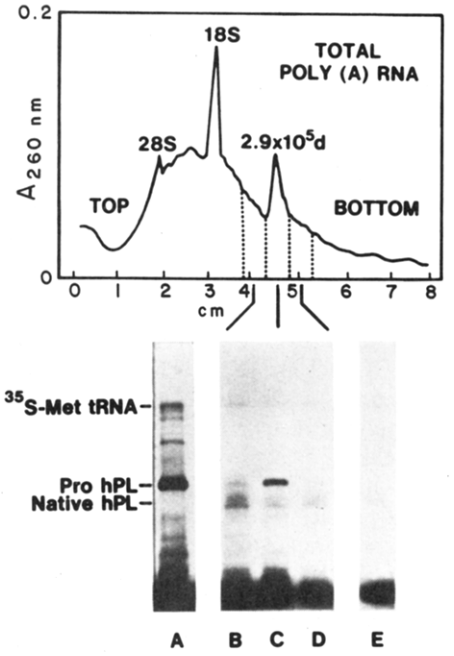

FIGURE 1: Absorbance profile (upper panel) of 2.75 μg of total poly(A+) mRNA on 1.3% agarose gel containing 5 mM CH_3HgOH and a fluorogram (lower panel) showing the products of in vitro translation of 1–2 μg of poly(A+) mRNA from (A) term placenta, (B) RNA immediately above the 2.9×10^5 dalton band ($\simeq$ 12 S), (C) RNA from the 12S band, (D) RNA immediately below the 12S band, and (E) the translation reaction mixture plus [^{35}S]-methionyl-tRNA incubated without added exogenous mRNA. Pro-hPL migrates as a 24 500-dalton band, and the mature hPL product migrates as a 21 500-dalton band. M_r of [^{35}S]methionyl-tRNA is 47 500 (R. Fehn, D. Gallo, and G. M. Lessard, personal communication, 1980).

column C), while total poly(A+) mRNA directed the synthesis of additional proteins of both lower and higher molecular weights (Figure 1, column A). The major protein was identified as pro-hPL by hPL-specific immunoprecipitation (Figure 1, column C). The precision of the isolation technique was demonstrated by the translation of mRNA recovered from gel slices immediately preceding (Figure 1, column B) and following (Figure 1, column D) the 290 000-dalton mRNA band. The products contained only negligible quantities of pro-hPL compared to those encoded by the selected RNA peak. On the basis of the synthesis of pro-hPL by the isolated pro-hPL mRNA, we estimated that its purification increased by ~115-fold over pro-hPL synthesized by total poly(A+) mRNA, as measured by hPL-specific immunoprecipitation methods [4.6×10^6 cpm/μg mRNA for pro-hPL synthesized with 2.9×10^5 dalton mRNA vs. 4×10^4 cpm/μg mRNA for pro-hPL synthesized with total poly(A+) mRNA]. The minor band preceding pro-hPL (Figure 1, column C) appears to migrate as hPL, which may have been released from pro-hPL by an endopeptidase activity in the translation reaction mixture, in agreement with other findings (Boime et al., 1976; Strauss et al., 1979). Cox et al. (1976) also observed that both pro-hPL and hPL were synthesized in the translation of placental poly(A+) mRNA in wheat germ extracts. We found that the mRNA trailing the 2.9×10^5 dalton band was predominantly translated into the mature product, while the 2.9×10^5 dalton mRNA was predominantly translated into pro-hPL. Therefore, the 2.9×10^5 dalton mRNA (pro-hPL mRNA) was the template used with avian myeloblastosis virus reverse transcriptase to yield the desired homogeneous pro-hPL cDNA probe ($R_0t_{1/2} = 8.6 \times 10^{-4}$ M·s) for the studies described below.

Table I: Protein and RNA Content of Various Human Placental Chromatin Fractions

constituents ratio	fractions		
	chromatin	DNA-S	DNA-P
histone:DNA	1.00	0	0
NHCP:DNA	1.00	0.05	0.54
RNA:DNA	0.24	0.026	0.38
% yield in DNA	100	96	4

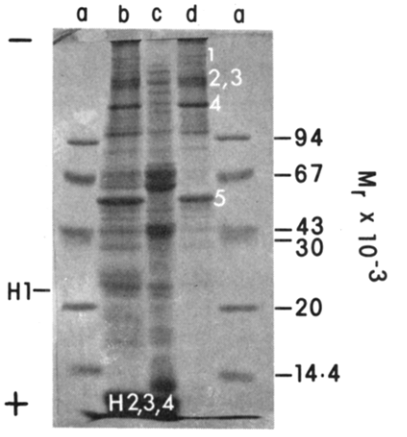

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoretic profile of (a) 2.5 μg of molecular weight marker proteins (from top to bottom: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and γ-lactalbumin) and 150 μg each of (b) total chromosomal proteins from purified chromatin, (c) 2 M NaCl soluble chromosomal proteins, and (d) tightly bound (DNA-P associated) chromosomal proteins. In arabic numerals on right of (d) we show positions of the five major high molecular weight proteins discussed in the text. H1, 2, [^{35}S]methionyl-tRNA and 4 designate histone bands.

Distribution of hPL Gene Sequences in DNA-P and DNA-S. DNA-P and DNA-S isolated from human placental chromatin exhibit variable protein:DNA and RNA:DNA ratios as shown in Table I. DNA-S is depleted of nonhistones, histones, and RNA, while DNA-P shows a ratio of nonhistones to DNA of ~0.5, is totally depleted of histones (Figure 2), and is slightly enriched in RNA content. The yield in DNA-P, on the average, is ~4% of total nuclear DNA. The nonhistone proteins in DNA-P consist of higher molecular weight chromosomal proteins found in chromatin, while lower molecular weight proteins including the histones are found in the 2 M NaCl soluble chromosomal proteins (Figure 2). Five major proteins (M_r 180 000, 150 000, 136 000, 120 000, and 58 000) are found in both DNA-P and chromatin. Proteins of M_r greater than 180 000 are also found exclusively in DNA-P and chromatin. Proteins of M_r between 60 000 and 80 000 and below 30 000 appear to be depleted from DNA-P but present in chromatin and in the 2 M NaCl soluble proteins. In the 2 M NaCl soluble chromosomal proteins, we find three major components which appear to be concentrated in this fraction (M_r 67 000, 60 000, and 43 000) but are absent from DNA-P. These proteins, however, are found in chromatin at concentrations approximating that of histone H1 (Figure 2). Therefore, both qualitative and quantitative differences are evident in the nonhistone protein composition of chromatin, DNA-P, and the 2 M NaCl soluble chromosomal proteins originating from the same tissue (Figure 2). If we assume that some of these differences observed in NHCP composition reflect different types of protein associations with DNA, then the data would suggest the existence of variability in the base sequence complexity between DNA-P and DNA-S. However, some dif-

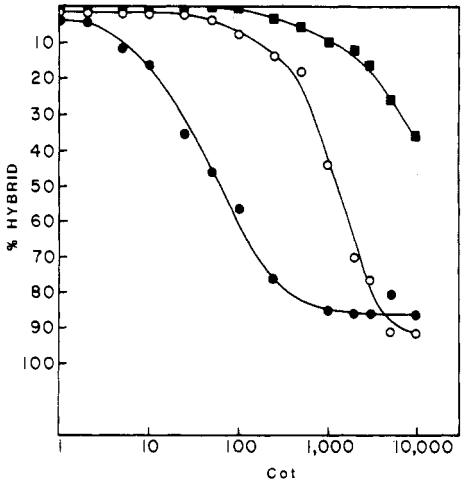

FIGURE 3: Reassociation kinetics of hPL [^{3}H]cDNA to (○) unfractionated human placental DNA (DNA-T), (●) DNA-P, and (■) DNA-S.

Table II: Distribution of hPL Gene Sequences in Different Fractions of DNA[a]

sample	$C_0t_{1/2}$	x-fold enrichment	% of total DNA	% hPL sequences[b]
DNA-T	1000	1.0	100	100
DNA-S	21000[c]	0.045 (or −22.2×)	96	4.4
DNA-P	45	22.2	4.1	91
DNase I[d]	20000[c]	0.05 (or −20×)	84	4.2
m. nucl.[d]	2000	0.50	60	30

[a] Data calculated from Figure 3. [b] % hPL DNA sequences = % total DNA × x-fold enrichment (Bloom & Anderson, 1978), where x-fold enrichment = $C_0t_{1/2}$ of DNA-T/$C_0t_{1/2}$ of DNA fraction. [c] Estimate based on extrapolation of curve. [d] Data calculated from Figure 4 and refer to DNA isolated from DNase I and micrococcal nuclease treated nuclei (see Experimental Procedure).

ferences may be due to nonspecific protein–protein interactions occurring in our preparations. Previously, we have demonstrated that DNA-P isolated from chicken reticulocyte chromatin was enriched 20-fold in sequences for the actively transcribed globin gene while being totally depleted from DNA-S (Bekhor & Mirell, 1979). Similarly, possible differences in the distribution of hPL gene sequences in DNA-P and DNA-S can be assessed by reassociation of [^{3}H]cDNA transcribed from purified pro-hPL mRNA with the two DNA fractions.

Since the rate of reassociation of hPL cDNA to DNA-P and DNA-S is dependent on the concentration of the hPL gene sequences in each DNA fraction, any depletion or enrichment in hPL gene sequences will be reflected in a respective increase or decrease in the observed $C_0t_{1/2}$ values. Figure 3 illustrates the striking differences in the hPL gene content of the different DNA fractions. The rapid reassociation of hPL cDNA with DNA-P demonstrates that DNA-P contains vastly more hPL gene sequences than unfractionated DNA (Figure 3; Table II), while the very slow and incomplete reaction of DNA-S with hPL cDNA (estimated $C_0t_{1/2}$ = 22 000 M·s) indicates that DNA-S is highly depleted of hPL gene sequences. We estimate that the DNA-P fraction, representing ~4% of the total nuclear DNA, is enriched ~22-fold and contains ~91% of the hPL gene sequences (Table II). In contrast, DNA-S, representing ~96% of the total nuclear DNA, contains only 4% of the hPL gene sequences, corresponding to an estimated depletion of 22-fold (Table II). Since the DNA-P was isolated

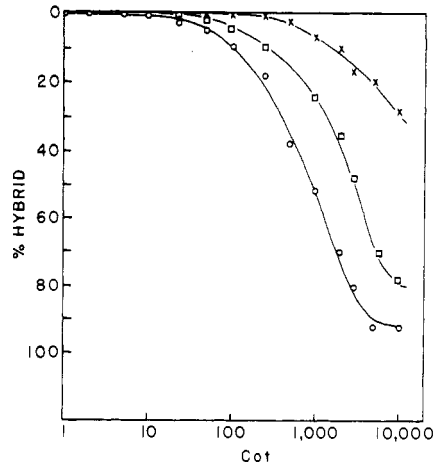

FIGURE 4: Reassociation kinetics of hPL [^{3}H]cDNA to (○) DNA-T, (×) DNA from nuclei treated with DNase I to 15% digestion, and (□) DNA from nuclei treated with micrococcal nuclease to a digestion level of 40%.

by a method based on precipitation with tightly associated proteins (see Experimental Procedure), the data of Figure 3 strongly suggest that the nature of the sequences in DNA-P may be determined by the tightly bound (2 M NaCl insoluble) chromosomal proteins.

Since the hPL gene is highly active in term placenta (Boime et al., 1976) and its mRNA can represent as much as 20% of the total poly(A+) mRNA (McWilliams et al., 1977), its sensitivity to digestion with DNase I was also investigated to provide a basis for further analysis on the nature of the sequences found in DNA-P. It has been shown that DNase I may preferentially digest most and possibly all active gene sequences in various tissues (Weintraub & Groudine, 1976; Garel & Axel, 1976). The results in Figure 4 reveal that the hPL gene, like the globin gene in chicken reticulocytes (Weintraub & Groudine, 1976) and the ovalbumin gene in chick oviduct (Garel & Axel, 1976), is highly sensitive to digestion with DNase I. The kinetics of the reassociation of hPL cDNA with DNA from DNased nuclei (Figure 4; Table II) suggest that ~96% of the hPL gene sequences are destroyed by DNase I treatment. Digestion of nuclei with micrococcal nuclease resulted in the removal of 70% of hPL gene sequences, although 80% of hPL [^{3}H]cDNA hybridized to micrococcal nuclease digested DNA at a C_0t of 10 000 M·s. Therefore, random and excessive digestion of hPL DNA sequences by micrococcal nuclease occurred with nuclei, while preferential digestion of the active hPL gene sequences was observed with DNase I, in agreement with other results (Weintraub & Groudine, 1976).

Sequence Homology between DNA-P and DNA-S. While the results from the hPL reassociation experiments (Figure 3) demonstrates that DNA-P and DNA-S differed in their content of one specific gene sequence (hPL), we wished to also examine the extent of sequence heterogeneity between the DNA-P and DNA-S fractions. We labeled a sample of DNA-P by nick translation and probed both DNA-S and DNA-T for sequence complementarity with DNA-P. The results (Figure 5; Table III) showed an apparent saturation of DNA-S sequences to DNA-P when only 44% of the DNA-P had reassociated to DNA-S. These data indicated that 55% (fraction of DNA-P reassociated with DNA-S/fraction of DNA-P reassociated with DNA-T, at C_0t = 40 000 M·s) of DNA-P sequences were common to both DNA-P and DNA-S, while 45% (1.8% of the total genome) were present in only DNA-P. In addition, the results suggested that while DNA-S was primarily depleted in single-Copy sequences comple-

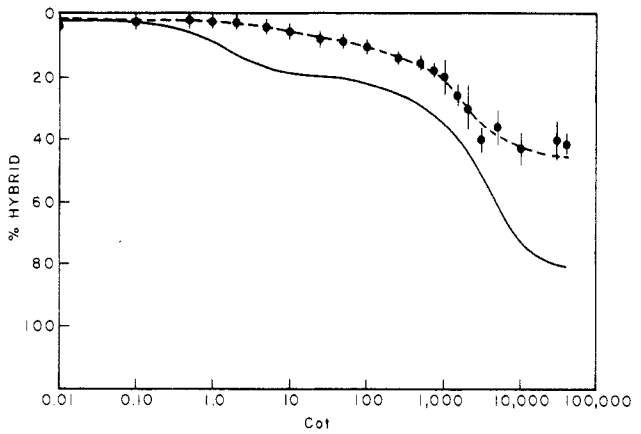

FIGURE 5: Reassociation kinetics of nick-translated [^{3}H]DNA-P to DNA-S (●) and to DNA-T (—). Bars indicate standard error between triplicate measurements.

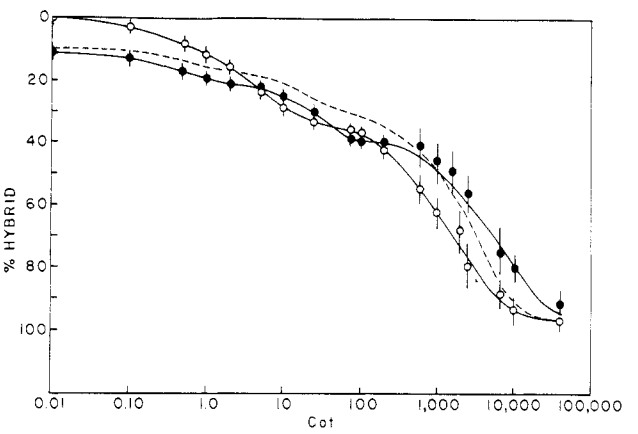

FIGURE 6: Reassociation kinetics of DNA-T (---), DNA-S (●), and DNA-P (○). Each DNA was nick translated as described under Experimental Procedure and reassociated with its homologous driver. Bars indicate standard error between triplicate measurements.

Table III: Percent of Nick-Translated [^{3}H]DNA-P Reassociated to Various DNA Fractions

DNA fraction	% at C_0t range (M·s) 0.01	0.01–1.0	1–100	100–10^5	% maximum hybridized
DNA-T[a]	0	8	14	58	80
DNA-S[a]	2	0	8	34	44
DNase I DNA[b]	0	0	16	37	53
m. nucl. DNA[b]	0	3	15	64	83

[a] Data calculated from Figure 5. [b] Data calculated from Figure 7 and refer to DNA isolated from DNase I and micrococcal nuclease treated nuclei (see Experimental Procedure).

Table IV: Percent of Hybrids Formed by Nick-Translated [^{3}H]DNA Fractions Reassociated with Homologous Driver[a]

DNA fraction	% at C_0t range (M·s) 0.01	0.01–1.0	1–100	100–10^5	$C_0t_{1/2}$ of unique class (M·s)
DNA-T	10	6	15	65	2500
DNA-S	12	8	18	58	5000
DNA-P	0	12	23	64	1000

[a] Data calculated from Figure 6.

mentary to the DNA-P probe, a significant component of repetitive sequences (C_0t between 1.0 and 100 M·s) present in the probe (Figure 6) were also depleted in DNA-S.

Sequence Organization of the Various DNA Fractions. The data presented in Figures 2–5 point to basic differences between DNA-P and DNA-S. We therefore examined the reassociation kinetics of each DNA fraction in comparison to the reassociation kinetics of total unfractionated DNA (DNA-T). The reassociation of unfractionated human DNA (Figure 6; Table IV) shows a minimum of two kinetic classes. Repetitive classes (C_0t < 100 M·s) comprise ~36% of the genome while the nonrepetitive sequences (C_0t > 100 M·s) make up the remaining 64% of the genome. These results are similar to estimates reported by others for human DNA (Saunders et al., 1972; Schmid & Deininger, 1975; Torelli et al., 1979). Comparison of the reassociation kinetics of DNA-S to those of DNA-T shows similarity of sequence organization (which is to be expected since DNA-S represents 96% of the total DNA) in which the primary difference is a minor depletion of single-copy sequences. In contrast, DNA-P is found to be enriched in both single-copy sequences (C_0t > 100 M·s) and moderately repetitive sequences (C_0t between 0.01 and 100 M·s). DNA-P is further distinguished from DNA-T and DNA-S fractions by its apparent total lack of highly repetitive sequences at a $C_0t \leq 0.01$ M·s., in contrast to the 11% content of these sequences in DNA-T and DNA-S. These findings support the hypothesis that a DNA fraction enriched in active genes should show both reduced complexity of the nonrepetitive DNA and total depletion of satellite DNA (Lewin, 1980).

Sensitivity of DNA-P to Digestion with DNase I. The data in Figure 5 showed that ~1.8% of the total genome was specifically located in the DNA-P fraction. This suggested that in addition to hPL gene sequences, other sequences might also be preferentially distributed between DNA-S and DNA-P. To determine the proportion of sequences in DNA-P which might be sensitive to digestion with DNase I, we digested placental nuclei with DNase I and subsequently probed the digested DNA for nuclease-resistant DNA-P sequences with the nick-translated [^{3}H]DNA-P probe. Micrococcal nuclease was used as a control for the nuclease digestions. The results in Figure 7, and as summarized in Table III, show that DNase I digestion hydrolyzed sequences complementary to ~27% (equivalent to 1.08% of the total genome) of the DNA-P probe, suggesting that this proportion of DNA-P may contain numerous active sequences in addition to hPL gene sequences. In contrast, DNA from nuclei digested with micrococcal nuclease showed essentially no depletion of sequences complementary to the DNA-P probe, although micrococcal nulcease treatment of nuclei did result in random digestion of the hPL gene (Figure 4). Most of the sequences which were removed by digestion with DNase I appear to be of the nonrepetitive type (21%, as shown in Table III; while 58% of DNA-P reassociating with DNA-T was nonrepetitive, only 37% of DNA-P reassociating with DNA from nuclei treated with DNase I was nonrepetitive).

Distribution of Total Poly(A+) mRNA Gene Sequences between DNA-P and DNA-S. It is clear from our data that many active gene sequences may be concentrated in DNA-P, while other active gene sequences may also be distributed in DNA-S. To evaluate the distribution of actively expressed sequences between DNA-S and DNA-P, we synthesized a cDNA probe from total poly(A+) mRNA isolated from placenta as described under Experimental Procedure and reassociated this probe to unfractionated DNA, DNA-P, and DNA-S. The results (Figure 8; Table V) show that 57% of this mixed cDNA probe reassociated with DNA-P while 58% reassociated with DNA-S. Although the results clearly demonstrated that DNA-P was enriched in poly(A+) mRNA gene

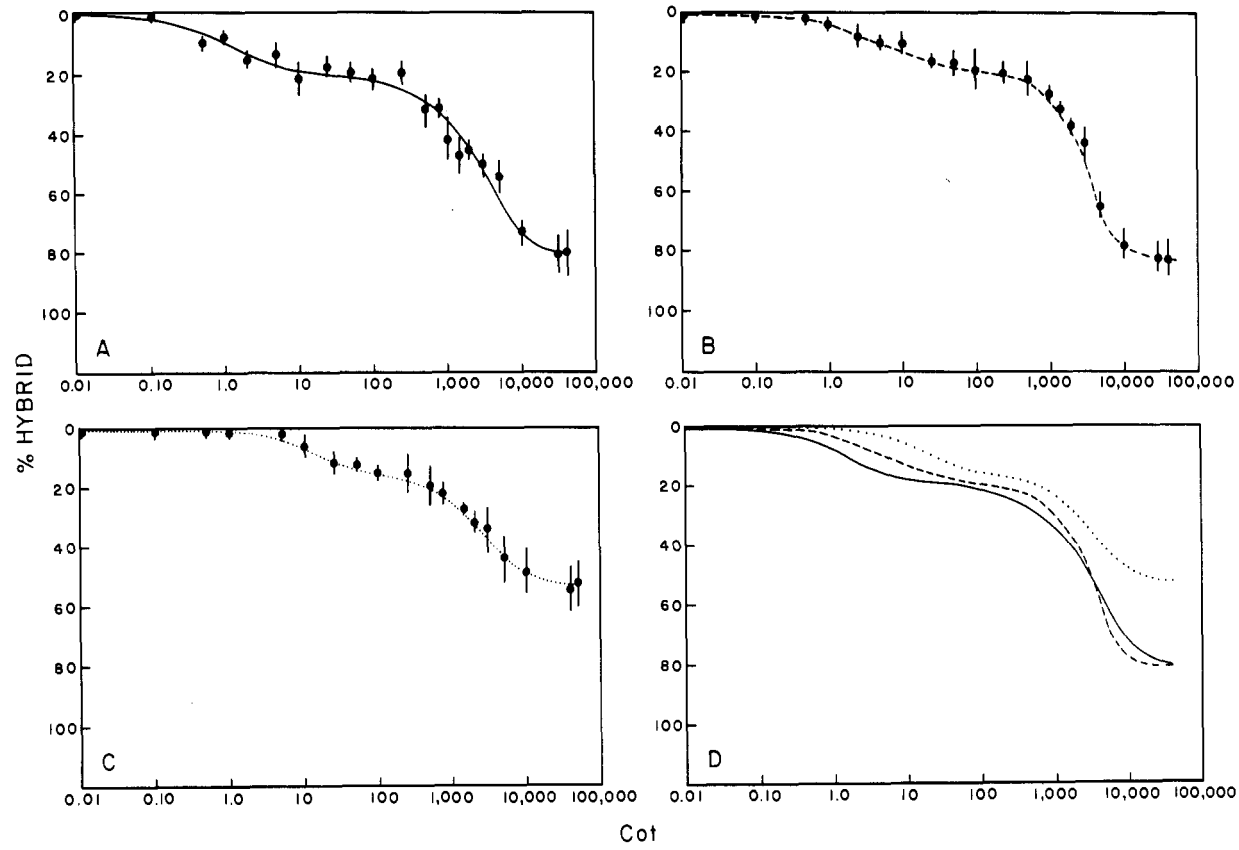

FIGURE 7: Reassociation kinetics of nick-translated [^{3}H]DNA-P to (A) DNA-T, (B) DNA from micrococcal nuclease treated nuclei, (C) DNA from DNase I treated nuclei, and (D) composite of curve A (—), curve B (---), and curve C (•••). Bars indicate standard error between triplicate samples.

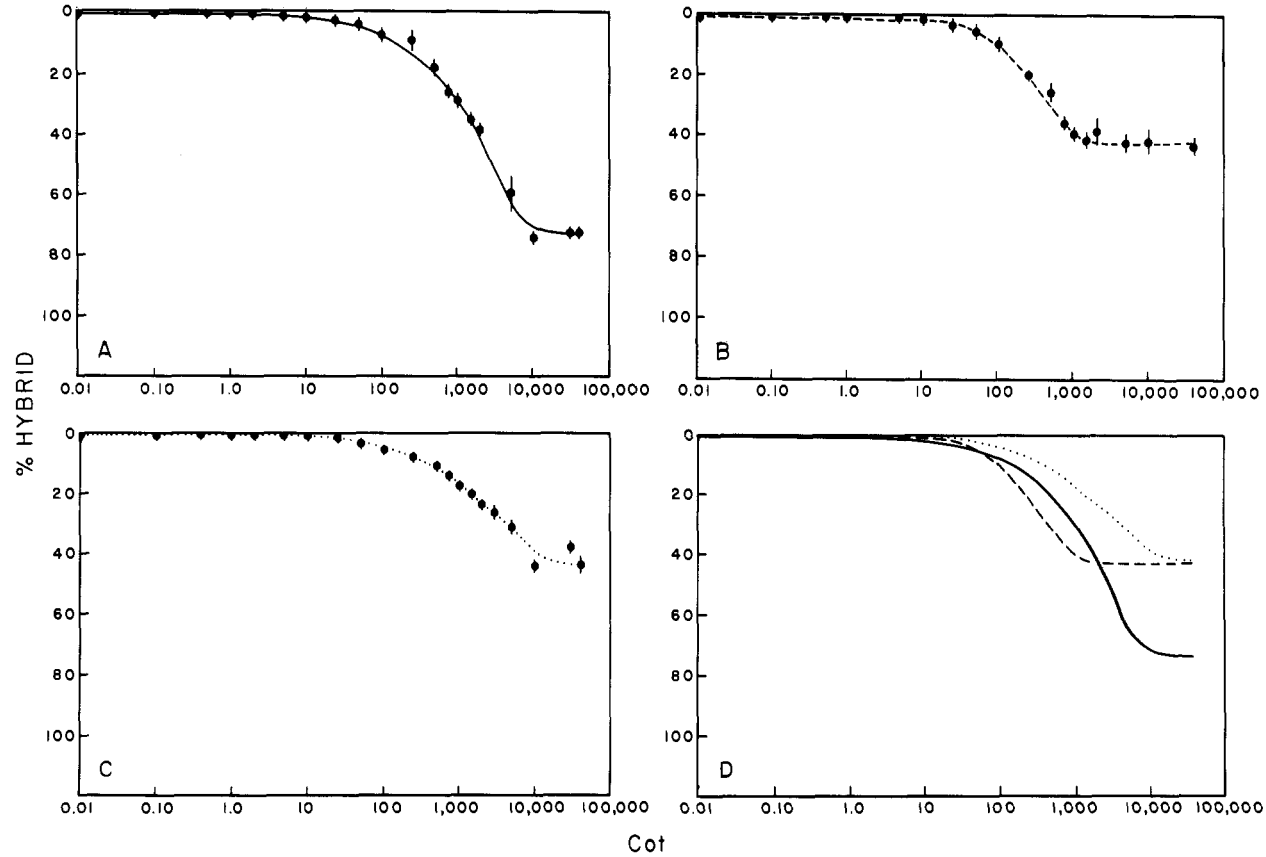

FIGURE 8: Reassociation kinetics of [^{3}H]cDNA transcribed from total placental poly(A+) mRNA to (A) DNA-T, (B) DNA-P, (C) DNA-S, and (D) composite of curve A (—), curve B (---), and curve C (•••). Bars indicate standard error between triplicate measurements.

sequences, the data were not sufficient to determine if the same population of cDNA had reassociated to both DNA-S and DNA-P or if DNA-P and DNA-S reassociated to different populations of cDNA sequences. To resolve the question of poly(A+) mRNA sequence distribution between DNA-P and DNA-S, we mixed purified DNA fractions isolated from

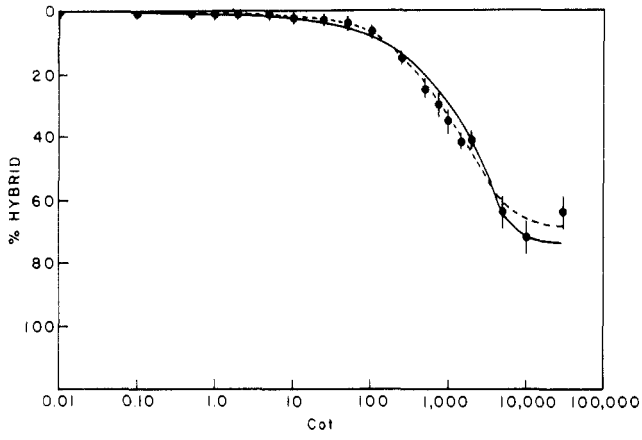

FIGURE 9: Reassociation kinetics of [^{3}H]cDNA transcribed from total placental poly(A+) mRNA to DNA-T (—) and to a DNA driver consisting of 4 parts of DNA-P and 96 parts of DNA-S (---). Bars indicate standard error between triplicate samples. Data for DNA-T were taken from Figure 8 (panel A).

DNA-P and DNA-S in the proportions which were found in chromatin (4% DNA-P plus 96% DNA-S) and then reassociated this mixture to the mixed cDNA probe. We reasoned that if DNA-P contained poly(A+) mRNA gene sequences which were absent in DNA-S, the addition of a "physiological" amount of DNA-P containing these sequences to DNA-S (at the ratio of 4:96) might reconstitute the full complement of poly(A+) mRNA gene sequences in the driver DNA. In contrast, if poly(A+) mRNA sequences in DNA-P were identical with those sequences already present in DNA-S, addition of DNA-P to DNA-S would cause little effect on the level of reassociation of DNA-P plus DNA-S to cDNA. The result of this experiment (Figure 9; Table V) demonstrated that 95% of the mixed cDNA probe reassociated with the mixed DNA driver, indicating that DNA-P and DNA-S contain different populations of poly(A+) mRNA gene sequences. Since DNA-S and DNA-P each contain sequences complementary to ~57% of the total poly(A+) mRNA sequences, it is apparent that these populations must have overlapping and nonoverlapping components. We estimate that 85% of the poly(A+) mRNA sequences are distributed equally and differentially between DNA-S and DNA-P, while ~15% of the poly(A+) mRNA sequences are common to both DNA-S and DNA-P. Also, the data of Figure 9 show that the reassociation curve for the mixed cDNA probe with DNA-P plus DNA-S appears to follow the same kinetics as that obtained for the reassociation of the mixed cDNA probe with unfractionated human placental DNA.

Discussion

Analysis of the DNA sequences associated with the 2 M NaCl insoluble chromosomal proteins (Figures 3–9) strongly suggests that a subset of active genes is preferentially located in these sequences. Three lines of evidence support this conclusion: (1) the hPL cDNA reassociation experiments demonstrated that 91% of the active hPL genes were located in DNA-P (Figure 3); (2) the DNase I sensitivity study (Figure 7) showed that 27% of DNA-P (equivalent to 1% of the total genome) was sensitive to digestion, implying that this proportion contained active gene sequences (Weintraub & Groudine, 1976); (3) reassociation kinetics of the cDNA probe transcribed from total poly(A+) mRNA (Figures 8 and 9) indicated that DNA-P and DNA-S each contained a nonoverlapping subset of active genes. Our results, therefore, suggest that the 2 M NaCl method permits the isolation of protein-bound DNA fragments which appear to be enriched

Table V: Reassociation Parameters of [^{3}H]DNA Transcribed from Total Placental Poly(A$^+$) Messenger RNA to Different DNA Fractions[a]

DNA fraction	$C_0t_{1/2}$ (M·s)	% maximum hybridized	x-fold enrichment
DNA-T	1800	74	1
DNA-S	1750	43	1.14
DNA-P	250	42	7.2
DNA-P:DNA-S (4:96)[b]	1500	70	1.20

[a] Data calculated from Figure 8. [b] Data calculated from Figure 9.

in active gene sequences. Electron microscopic visualization of DNA-P isolated from chicken reticulocytes has shown that the 2 M NaCl insoluble DNA, enriched in globin gene sequences (Bekhor & Mirell, 1979), is associated with nonhistone proteins at ~50–100 base-pair intervals (unpublished results), unequivocally supporting the conclusion that the DNA in DNA-P is indeed associated with proteins. Whatever the precise mechanism involved in the association of the tightly bound proteins in DNA-P, the empirical fact remains that a small subset of DNA is nonrandomly separated from the bulk of the genome exclusively as a result of its association with the tightly bound chromosmal proteins.

Although we are presently focusing our efforts on investigations of the tightly bound chromosomal proteins, it is clear that these proteins are probably only one of many elements involved in regulating genetic activity. A number of recent studies have demonstrated that the proteins HMG 14 and HMG 17, extractable from chromatin with 0.35 M NaCl, were essential for maintaining the DNase I sensitivity of the globin gene in chromatin from chicken reticulocytes (Weisbrod & Weintraub, 1979; Weisbrod et al., 1980) and erythrocytes (Sandeen et al., 1980). A similar type of a protein, H6, was found to be associated with DNase I sensitive chromatin regions of trout testis (Levy-Wilson et al., 1980). The low affinity of these proteins for chromatin argues against sequence specific binding, and the observed lack of tissue specificity of HMG 14 and HMG 17 (Weisbrod & Weintraub, 1979) supports this view. Chromatin that has been extracted with 1 M NaCl and reconstituted with HMG 14 and HMG 17 no longer exhibits sensitivity of active genes to DNase I (Weisbrod et al., 1980). These results support our assumption that additional components are necessary for selective interaction of HMG 14 and HMG 17 to chromatin. The dissociated 1 M NaCl components, while providing an increased level of specificity, might in turn require more tightly bound proteins to achieve their ultimate specificity. Our results on the distribution of the lactogen gene in human placental chromatin (Figure 3) suggest that the tightly bound proteins are preferentially associated with the active tissue-specific hPL gene. The observation that the tightly bound proteins interact with many active gene sequences, however (Figures 5–9), implies that these proteins may possess some sequence specificity for interactions with a number of genes and not only with one specific gene. Therefore, it may be postulated that the tightly bound proteins are able to interact with a number of physiologically related genes, and it is possible that the primary sequence of the DNA allows protein recognition of a group of genes which together may result in the expression of the cell phenotype. If this is correct, then mechanistically the majority of the proteins which we find tightly associated with DNA may constitute a *coarse* control of gene expression, while the *fine* control is encoded in the DNA base sequence itself.

The control of gene expression by these proteins is necessary, however, for correct genetic readout.

Considerable evidence indicates that tissue specificity lies in the tightly bound chromosomal proteins. Recently it was shown that the nonhistone protein components of the chromatin antigenic complex were very tightly bound to DNA (Pumo et al., 1980). Earlier, Chiu et al. (1975) reported that a tightly bound nonhistone protein fraction, consisting of ~5% of the total chromosomal proteins, was necessary for the efficient in vitro transcription of the chicken globin gene. Further work suggested that these tightly bound proteins were preferentially associated with "active–diffuse" rather than "inactive–condensed" chromatin (Wang et al., 1976). A tightly bound NHCP fraction designated AP3 has been isolated from chick oviduct by Spelsberg et al. (1976). This fraction, when bound to DNA, was found to contain high-affinity progesterone–receptor complex acceptor sites.

Extraction of metaphase and interphase nuclei with 2 M NaCl leaves a protein skeleton referred to as scaffolding proteins (Aldoph et al., 1977), nuclear cage (Cook & Brazell, 1980), or nuclear matrix (Berezney & Coffey, 1977). Electron micrographic examination of this residual protein structure reveals radial DNA loops extending out from a central axis (Marsden & Laemmli, 1979). It has been suggested that the loops are stabilized at their base by NHCP of the nuclear matrix structure (Cook & Brazell, 1975; Benyajati & Worcel, 1976; Igo-Kemenes & Zachau, 1977; Laemmli et al., 1977; Comings, 1980). What then is the relationship between the nuclear matrix and DNA-P?

The nuclear matrix is usually prepared by treating washed nuclei with DNase I at 4 °C for 30 min, followed by extracting with 2 M NaCl, and finally washing and resuspending in Tris–EDTA buffer resulting in the removal of ~98% of the DNA (Barrack & Coffey, 1980). It is expected that digestion of the nuclei with DNase I would result in the initial hydrolysis of the active genes, followed by degradation of resistant or inactive DNA (Weintraub & Groudine, 1976). Since DNA-P and the nuclear matrix are both prepared by extraction with 2 M NaCl, we would expect some similarities in their protein composition. In the case of the nuclear matrix, however, both chromosomal and nonchromosomal proteins will be present. The DNA-P fraction will be highly enriched in chromosomal protein since it is prepared from chromatin. The nuclear matrix appears therefore as a residual nuclear protein structure containing RNP particles (Miller et al., 1978a) with remnants of chromatin. Characterization of the remaining DNA (0.1–0.5% of the total DNA) still bound to metaphase chromosomes after exhaustive digestion with DNase I has indicated that these sequences were primarily of the highly repetitive type (Jeppeson & Bankier, 1979). Preparation of a protein skeleton by treating interphase and metaphase nuclei with both micrococcal nuclease and restriction endonucleases results in a DNA (3–5% of the total) that is enriched in middle repetitive sequences (Razin et al., 1979). Therefore, if the matrix is associated with the middle repetitive sequences, then there is the possibility of overlap between the middle repetitive DNA-P sequences and the matrix-bound middle repetitive sequences. If only highly repetitive sequences are present, however, then there appears to be no overlap, since we find DNA-P to be devoid of these sequences (Figures 5–7). The presence of highly repetitive DNA sequences in the nuclear matrix would suggest that this residual DNA may have originated from heterochromatin.

A number of other observations have implicated the nuclear matrix in various nuclear functions. Preparations utilizing DNase I digestion contain binding sites for steroid hormones (Barrack & Coffey, 1980), hnRNA, and small nuclear RNA (Miller et al., 1978a,b) and attachment sites for chromatin during replication (Dijkwel et al., 1979). When restriction endonucleases replace DNase I in the preparation of the nuclear matrix, the DNA attached to protein is found enriched in the α-globin gene in HeLa cells (5-fold; Cook & Brazell, 1980) and in ovalbumin gene in laying hen oviduct (3-fold; M. T. Kuo, personal communication, 1980). Consideration of the methodology used and the results obtained suggests that it is possible that a part of these nuclear functions might be specifically located in the DNA-P fraction rather than in the non-chromatin-derived segment of the matrix.

The concept of looped chromosomal domains presents an appealing model in terms of both structural and biochemical observations (Laemmli et al., 1977). Since DNA-P appears to be highly involved (up to 27% of its sequences) in active transcription, one could easily speculate that the DNA-P fragments originate from the looped structures, where the loops, like those in lampbrush chromosomes, are involved in transcription. The method of isolation of DNA-P necessitates considerable shearing in order to concentrate the DNA-P sequences. Therefore, very large fragments of DNA, on the order of a domain length (50–100 kbp), would probably be difficult to obtain. Further analysis must be done to clarify the relationship of DNA-P to these structures.

Several features of DNA-P deserve particular discussion. First, the 22-fold enrichment of DNA-P for the very active human placental lactogen gene sequences (Figure 3) immediately indicated that the DNA-P sequences represent a highly nonrandom subset of the genomic DNA. In addition, the observation that DNA-P contains sequences complementary to 57% of the total poly(A+) mRNA, with 43% of the total poly(A+) mRNA complementary sequences represented exclusively in DNA-P (Figure 8), further indicates the selectivity of the sequences found in DNA-P. We have previously suggested (Bekhor & Mirell, 1979) that the tightly bound proteins may be preferentially associated with cell-specific gene sequences. This observation appears to hold for both the chicken globin gene in reticulocytes and the human lactogen gene in placenta. Finally, DNA-P is enriched in single copy, slightly enriched in middle repetitive, but totally depleted of highly repetitive sequences. This finding suggests that the highly repetitive sequences are probably not extensively involved in active expression in DNA-P.

The DNA-S fraction also exhibited a significant content of active genes (Figure 8). The nature of these genes and their mode of regulation are at present unknown. In conclusion, we have demonstrated that a group of NHCP's are tightly associated with a nonrandom component of the human genome enriched in selected active sequences. The association of the proteins with these sequences suggests a likely role in gene expression.

References

Adolph, K. W., Cheng, S. M., & Laemmli, U. K. (1977) *Cell (Cambridge, Mass.) 12*, 805–816.

Allen, E., & Schweet, L. (1962) *J. Biol. Chem. 237*, 760–767.

Bailey, J. H., & Davidson, N. (1976) *Anal. Biochem. 70*, 75–85.

Balmain, A., & Birnie, G. D. (1979) *Biochim. Biophys. Acta 561*, 155–166.

Bantle, J. A., Maxwell, I. H., & Hahn, W. E. (1976) *Anal. Biochem. 72*, 413–427.

Barrack, E. R., & Coffey, D. S. (1980) *J. Biol. Chem. 255*, 7265–7275.
Bekhor, I., & Samal, B. (1977) *Arch. Biochem. Biophys. 179*, 537–544.
Bekhor, I., & Mirell, C. J. (1979) *Biochemistry 18*, 609–616.
Benyajati, C., & Worcel, A. (1976) *Cell (Cambridge, Mass.) 9*, 393–407.
Berezney, R., & Coffey, D. S. (1977) *J. Cell Biol. 73*, 616–637.
Bloom, K. S., & Anderson, J. N. (1978) *Cell (Cambridge, Mass.) 15*, 141–150.
Boime, I., McWilliams, D., Szczesna, E., & Camel, M. (1976) *J. Biol. Chem. 251*, 820–825.
Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.
Bradford, N. M. (1976) *Anal. Biochem. 72*, 248–254.
Burton, K. (1955) *Biochem. J. 62*, 315–323.
Campbell, A. M., Briggs, R. C., Bierd, R. E., & Hnilica, L. S. (1979) *Nucleic Acids Res. 6*, 205–218.
Case, S. T., & Daneholt, B. (1976) *Anal. Biochem. 74*, 198–206.
Chiu, J.-F., Tsai, Y.-H., Sakuma, K., & Hnilica, L. S. (1975) *J. Biol. Chem. 250*, 9431–9433.
Chytil, F., & Spelsperg, T. C. (1971) *Nature (London), New Biol. 233*, 215–218.
Comings, D. (1980) *Hum. Genet. 53*, 131–143.
Cook, R., & Brazell, I. A. (1975) *J. Cell Sci. 19*, 261–279.
Cook, R., & Brazell, I. A. (1980) *Nucleic Acids Res. 8*, 2895–2906.
Cox, G. S., Weintraub, B. D., Rosen, S. W., & Maxwell, E. S. (1976) *J. Biol. Chem. 254*, 1723–1730.
David, A. W., & Phillips, D. R. (1978) *Biochem. J. 173*, 179–183.
Defer, N., Kitzis, A., Levy, F., Tichonicky, L., Sabatier, M.-M., & Kruth, J. (1978) *Eur. J. Biochem. 88*, 583–591.
Dijkwel, P. A., Mullenders, L. H. F., & Wanka, F. (1979) *Nucleic Acids Res. 6*, 219–230.
Dische, Z., & Schwarz, K. (1937) *Mikrochim. Acta 2*, 13–19.
Friedman, E. Y., & Rosbash, M. (1977) *Nucleic Acids Res. 4*, 3455–3471.
Garel, A., & Axel, R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3966–3970.
Gates, D. M., & Bekhor, I. (1980) *Science (Washington, D.C.) 207*, 661–662.
Goodwin, G. H., Mathew, C. G. P., Wright, C. A., Venkor, C. D., & Johns, E. W. (1979) *Nucleic Acids Res. 7*, 1815–1835.
Handwerger, S., & Sherwood, L. M. (1974) in *Methods of Hormone Radioimmunoassay* (Jaffe, B. M., & Behrman, H. R., Eds.) pp 417–426, Academic Press, New York.
Hartree, E. F. (1972) *Anal. Biochem. 48*, 422–427.
Helling, R. B., Goodman, H. M., & Boyer, H. (1974) *J. Virol. 14*, 1235–1244.
Igo-Kemenes, W. T., & Zachau, H. G. (1977) *Cold Spring Harbor Symp. Quant. Biol. 42*, 109–118.
Jeppeson, P. G. N., & Bankier, A. T. (1979) *Nucleic Acids Res. 7*, 49–67.
Kessler, S. N. (1975) *J. Immunol. 115*, 1617–1624.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Laemmli, U. K., Cheng, S. M., Aldolph, K. W., Paulson, J. R., Brower, J. A., & Baumbach, W. R. (1977) *Cold Spring Harbor Symp. Quant. Biol. 42*, 351–360.
Lapeyre, J.-N., & Bekhor, I. (1976) *J. Mol. Biol. 104*, 25–58.
Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335–341.
Levy-Wilson, B., Kuchl, L., & Dixon, G. H. (1980) *Nucleic Acids Res. 8*, 2859–2869.
Lewin, B. (1980) in *Gene Expression* (Lewin, B., Ed.) Vol. 2, Wiley, New York.
Liew, C. C., & Chan, P. K. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3458–3462.
Louie, A. J., & Dixon, G. H. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1975–1979.
Marsden, M. P. F., & Laemmli, U. K. (1979) *Cell (Cambridge, Mass.) 17*, 849–858.
Martial, J. A., Baxter, J. A., & Goodman, H. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1816–1820.
McGhee, J. D., & Felsenfeld, G. (1980) *Annu. Rev. Biochem. 49*, 1115–1156.
McWilliams, D., Callahan, R. C., & Boime, I. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1024–1027.
Miller, T. E., Huang, C.-Y., & Pogo, A. O. (1978a) *J. Cell Biol. 76*, 675–691.
Miller, T. E., Huang, C.-Y., & Pogo, A. O. (1978b) *J. Cell Biol. 76*, 692–704.
Pelham, H. R. B., & Jackson, R. S. (1976) *Eur. J. Biochem. 67*, 247–256.
Ponder, B. A. J., & Crawford, L. V. (1977) *Cell (Cambridge, Mass.) 11*, 35–49.
Pribnow, D. (1979) in *Biological Regulation and Development* (Goldberger, R. F., Ed.) Vol. 1, pp 219–277, Plenum, New York.
Pumo, D. E., Wierzbicki, R., & Chiu, J.-F. (1980) *Biochemistry 19*, 2362–2367.
Razin, S. V., Mantieva, V. L., & Georgiev, G. P. (1979) *Nucleic Acids Res. 7*, 1713–1735.
Rowe, D. W., Moen, R. C., Davidson, J. M., Byers, P. H., Bornstein, P., & Palmiter, R. D. (1978) *Biochemistry 17*, 1581–1590.
Ruiz-Carrillo, A., Wangh, L. J., & Allfrey, V. G. (1975) *Science (Washington, D.C.) 197*, 117–128.
Sandeen, G., Wood, W. I., & Felsenfeld, G. (1980) *Nucleic Acids Res. 8*, 3757–3778.
Saunders, G. F., Shirakawa, S., Saunders, P. P., Arrighi, F. E., & Hsu, T. C. (1972) *J. Mol. Biol. 63*, 323–334.
Schmid, C. W., & Deininger, P. C. (1975) *Cell (Cambridge, Mass.) 6*, 345–358.
Spelsberg, T. C., Webster, R., Pikler, G., Thrall, C., & Wells, D. (1976) *J. Steroid Biochem. 7*, 109–1101.
Strauss, A. W., Zimmerman, M., Boime, I., Ashe, B., Mumford, R. A., & Alberts, A. W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4225–4229.
Torelli, G., Cadossi, G., Ferrari, S., Narmi, F., Ferrari, S. Montegnani, G., Torelli, U., & Bosi, P. (1979) *Biochim. Biophys. Acta 561*, 301–311.
Tsai, S. Y., Harris, S. C., Tsai, M.-J., & O'Malley, B. W. (1976) *J. Biol. Chem. 251*, 4713–4721.
Wang, S., Chiu, J.-F., Klyszejko-Stefanowicz, L., Fujitani, H., & Hnilica, L. S. (1976) *J. Biol. Chem. 251*, 1471–1475.
Wang, T. Y. (1978) *Biochim. Biophys. Acta 518*, 81–88.
Weintraub, H., & Groudine, M. (1976) *Science (Washington, D.C.) 193*, 848–856.
Weisbrod, S., & Weintraub, H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 630–634.
Weisbrod, S., Groudine, M., & Weintraub, H. (1980) *Cell (Cambridge, Mass.) 19*, 289–310.
Witting, P., & Wittig, S. (1979) *Cell (Cambridge, Mass.) 18*, 1173–1183.