Nucleoprotein hybridization: a method for isolating active and inactive genes as chromatin

Claudius Vincenz, Jan Fronk⁺, Graeme A. Tank and John P. Langmore^{*}

Biophysics Research Division, University of Michigan, 2200 Bonisteel Boulevard, Ann Arbor, MI, 48109-2099, USA

Received August 14, 1990; Revised and Accepted February 9, 1991

ABSTRACT

The developmentally regulated sea urchin early histone gene repeat (SUEHGR) from *Strongylocentrotus purpuratus* was isolated as chromatin by nucleoprotein hybridization. This technique is a novel method to isolate specific sequences as chromatin. Because the purification scheme is based only on the gene sequence and is independent of other physical properties such as protein composition and transcriptional activity, we were able to isolate the same gene in different functional states. Gene size chromatin fragments were solubilized by restriction endonuclease digestion of cell nuclei. Using T7 gene 6 exonuclease, the 3' termini of the fragments were exposed and then hybridized in solution to a biotinylated oligonucleotide complementary to one end of the SUEHGR fragment. The hybrids were bound to an Avidin D matrix. DTT cleavage of the biotin linker yielded a chromatin fraction >700 fold enriched in SUEHGR. Overall yields were between 2% and 15%. The purity of the isolated material was independently measured to be >80%. The homogeneous native structure of the inactive genes was preserved as shown by electron microscopy and micrococcal nuclease digestion of the purified SUEHGR. Minor heterogeneity was observed for the purified active genes by micrococcal nuclease digestion but the main features of the active chromatin were preserved during isolation. This isolation offers the first opportunity to study the structure of an RNA polymerase II gene at different stages of the cell cycle and development.

INTRODUCTION

Characterization of proteins and other molecules associated *in vivo* with a particular gene has been difficult due to the complexity of the eukaryotic genome and the physical properties of chromatin. Many approaches to investigate proteins bound to chromatin have been attempted, such as reconstitution (e.g. 1–5), indirect assays for protein binding (e.g. 6–10), and chromatin

fractionation (e.g. 11–25). None of these procedures have been able to identify the full spectrum of proteins bound to a single gene in different functional states.

The nucleoprotein hybridization technique is unique in its potential to purify a chromatin fragment with a specific sequence irrespective of its functional state. The feasibility of the technique was proven with an SV40 model system (26). SV40 minichromosomes were mixed with an excess of exogenous sea urchin chromatin and then isolated to 88% purity corresponding to a 115 fold enrichment. We present here the first purification of specific cellular genes as chromatin, based on the same principle of specific DNA-chromatin hybridization.

The purification scheme uses the specificity of restriction enzymes and a 5' exonuclease to generate chromatin fragments with short (<100 bases) single-stranded ends. A biotinylated oligonucleotide is synthesized complementary to one end of the targeted gene. Specific hybridization between the oligonucleotide and the exposed chromatin tail is performed in solution. Affinity chromatography is then carried out using an avidin matrix. The hybrids are eluted by cleaving a disulfide bond located in the linker of the biotin analog. The salt and temperature conditions throughout the procedure are compatible with intact chromatin.

The *Strongylocentrotus purpuratus* sea urchin early histone gene repeat (SUEHGR) was chosen as a target for the application of the technique for two reasons. First, the SUEHGR is a unit of 6.5 kb which is tandemly repeated head-to-tail about 500 times in the genome (27). This reduces the amount of nuclei and enzymes needed to purify amounts large enough for biochemical analysis. The high copy number also reduces the enrichment necessary to obtain pure material. Second, the SUEHGR changes its transcriptional activity during early development in a well established fashion (28). The genes are transcriptionally dormant in the early zygote, become activated at early morula stage, reach a maximum transcriptional rate around the 64–128 cell stage (corresponding to 8–10 h of development under standard laboratory conditions), and then gradually become inactivated at the hatching blastula stage (18 h). These genes are a suitable model for the majority of cellular genes because they are transcribed at a similarly moderate rate (2–3 transcripts per gene per minute; ref. 28). Studies on specific chromatin fragments

* To whom correspondence should be addressed

⁺ Present address: Institute of Biochemistry, Warsaw University, Zwirki I Wigury 93, 02089 Warsaw, Poland

were up to now only possible on genes with unusual transcriptional rates (i.e. rRNA genes, ref. 29–31; and chorion genes, ref. 32).

Here we report the purification of the SUEHGR chromatin fragment by nucleoprotein hybridization. Independent assays are described for the critical steps of the procedure. The final enrichment of SUEHGR was >700 fold. The overall yield was 2–15%. The purity measured by two independent methods was >80%. The micrococcal nuclease digestion patterns for the isolated SUEHGR were identical to those for the nuclei prior to isolation. Electron microscopy (EM) of low salt spreads confirmed that the isolated material was chromatin. Previous results showing the absence of core histone exchange under nucleoprotein hybridization conditions (26) were confirmed. The results show promise of being able to determine the histone variants and post translational modifications that are associated with the different functional states of multi-copy genes such as the SUEHGR.

MATERIALS AND METHODS

Materials

Vectrex™ Avidin affinity matrix was from Vector Laboratories, Inc. Burlingame, CA. Sulfosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate (NHS-SS-Biotin) was purchased from Pierce. Glutathione (disodium salt, oxidized form Grade IV, GSSG), leupeptin (hemisulfate salt) and N-lauroylsarcosine (sarkosyl) were from Sigma. High concentration SalI (70 U/μl) was from Boehringer Mannheim. All other restriction enzymes, λ exonuclease and T4 kinase were from Bethesda Research Laboratories. T7 gene 6 exonuclease was from United States Biochemical Corporation. Zeta-Probe nylon membrane was from Bio-Rad. [³²P] dCTP, [methyl-³H] thymidine and L-[³⁵S]methionine were from Amersham. The following solutions were used: SSPE, SSC, TAE and TE were as in Maniatis (33). Synthetic sea water; 1 liter contains 24.72 g of NaCl, 0.67 g of KCl, 1.36 g of CaCl₂·2H₂O, 4.66 g of MgCl₂·6H₂O, 6.29 g MgSO₄·7H₂O, and 1.8 g NaHCO₃. Buffer A; 15 mM Hepes pH 7.3, 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 0.5 mM spermidine, and 0.15 mM spermine. Chromatin digestion buffer, buffer D; 50 mM NaCl, 50 mM Tris pH 8.0, 3 mM MgCl₂, and 1 mM 2-mercaptoethanol. Chromatin hybridization buffer, buffer H; 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 0.05% NP-40, and 0.02% azide. Affinity chromatography buffer, buffer C; buffer H containing 5 mM GSSG. Homogenization buffer, buffer Ho; buffer A containing 0.3 M sucrose, 5 mM iodoacetate, and 0.5 mM PMSF.

Synthesis and purification of the biotinylated oligonucleotides

Oligonucleotides were synthesized at the University of Michigan DNA facility on an Applied Biosystems Model 391 DNA synthesizer and aminomodified with aminolink 2 from Applied Biosystems according to the instructions of the manufacturer. The aminomodified oligonucleotide was cleaved from the support, HPLC purified, and deprotected. Biotinylation was done at room temperature with 10 μg of 17^{mer} dissolved in 450 μl of 0.2 M Hepes pH 7.7 to which 6 mg solid NHS-SS-Biotin (Pierce) was added and the reaction mixture was shaken for 1.5 h. The insoluble material was removed by centrifugation and the supernatant was purified in 2 batches with a reverse phase VYDAC C⁴ HPLC column (pore size 300 Å) using 0.1 M

triethylammonium chloride pH 7.0 as buffer and a 5–25% acetonitrile gradient in 20 min. Reanalysis of the isolated material on the same system gave a single peak eluting at 12% acetonitrile.

Sea urchin embryo cultures and nuclear isolations

The sea urchins (*Strongylocentrotus purpuratus*) were purchased from Alacritty Biomarine, Redondo Beach, CA. Gametes were collected by coelomic injection of 1 ml 0.5 M KCl. Eggs were filtered through a spectra/mesh 100 μm pore nylon filter. Sperm were collected directly with capillary pipettes. Up to 40 ml of eggs were fertilized in 250 ml synthetic sea water containing 1 mM 3-amino-1,2,4-triazole (34). Embryos were washed twice in 500 ml synthetic sea water (settling of the embryos was at 1 g) and resuspended in 1 liter synthetic sea water containing 100 mg streptomycin sulfate and then incubated at 15°C under gentle stirring. [³H]Thymidine (1 mCi) was added 5 min after the start of the incubation. For protein labeling 40 ml of fertilized eggs were incubated in a 250 ml volume to which [³⁵S]methionine (1 mCi) was added after 4.5 h of incubation. Embryo cultures were put on ice after the desired incubation time. Fertilization membranes were removed from 10 h embryos by filtration through a 52 μm pore spectra/mesh nylon filter. Embryos were washed at 1 g on ice for >4 h using 100 ml buffer Ho. A 2nd wash with 100 ml Ho. buffer was done at 50 g for 10 min. Embryos (24 ml) were homogenized with 25 strokes of a teflon pestle. Then Ho buffer (49 ml) containing 2.3 M sucrose was mixed in and 12 ml of this homogenate was layered over 1 ml buffer Ho containing 2.3M sucrose and centrifuged in a SW40 rotor for 35 min at 30,000 RPM. The opaque nuclear bands were collected and washed 3 times in buffer A containing 0.1% NP-40, 0.1 mM PMSF, and 5 mM iodoacetate (7.5 ml, 15ml, 10ml) and resuspended in the same buffer. Buffer A/80% glycerol was mixed in to a final glycerol concentration of 50% and a DNA concentration of 1 mg/ml. The suspension was quick frozen at –80 °C. Protein and DNA analysis revealed no degradation during isolation or after storage at –80°C for more than 1 year. The nuclei were intact as verified by phase contrast microscopy. Only nuclei derived from preparations with > 90% fertilization efficiency and normal development were used for nucleoprotein hybridization.

Chromatin solubilization

In a typical preparation 2 ml of nuclei were thawed on ice and diluted to 0.5 mg/ml with buffer D containing 12 μM leupeptin. The solution was transferred into a dialysis bag (spectra/por #2). HindIII (5 U/μg DNA) was added and dialysed 3 h at 37°C against 1 liter of buffer D. Additional leupeptin was added to 6 μM and dialysis continued 3 h against 1 liter buffer D containing 0.5 mM Mg. The nuclei were then slowly lysed by dialysis against 1 mM EDTA for >4 h at 4°C. Insoluble material was removed by 15 min centrifugation at 10,000 g. Solubilization efficiencies were determined by scintillation counting of aliquots. The nuclei were hydrolyzed 3 h in 10% trichloroacetic acid (TCA) 3 h at 90°C before counting to eliminate self quenching. The same protocol was followed for solubilization with SalI except that buffer D contained 100 mM NaCl during the first dialysis.

Exonuclease digestion

An equal volume (~6 ml) of 20 mM Tris pH 8.0, 2 mM MgCl₂, and 12 μM leupeptin was added to the solubilized chromatin followed by a 5 min centrifugation at 2000 g. Four units T7 gene 6 exonuclease per μg soluble chromatin was added

to the supernatant followed by a 30 min incubation at 37°C. An equal amount of exonuclease was added and the incubation continued for 30 min. Alternatively, λ exonuclease digestion was done in the same buffer with 5 U/ μ g for 3 h. The reaction was stopped by adding EDTA to 1 mM. TCA solubility was determined by adding carrier salmon sperm DNA (100 μ g/ml) and cold TCA to 6%. The supernatant was counted after 15 min centrifugation at 10,000 g.

Hybridization

The chromatin (typically 400 μ g in 12 ml) was concentrated 3–5 fold by dialysis for 3 h at 4°C against buffer H containing 60% sucrose. Leupeptin was added to 20 μ M, the clamps on the dialysis tubing were tightened, and the sucrose was removed by dialysis overnight at 4°C against buffer H. Next, 5 mM GSSG, 5 mM iodoacetamide (IAcNH₂), 6 μ M leupeptin, and 3.6 nM biotinylated oligonucleotide were added from concentrated stock solutions. The standard hybridization time was 24 h at 37°C after which the mixture was spun at 2000 g for 5 min.

Avidin D affinity chromatography

For a standard preparative isolation 110 mg of dry Vectrex Avidin D were used (4 mg per ng of 17^{mer}). The resin was reconstituted for 30 min in H₂O and then loaded into Bio Rad 2 ml Poly-Prep columns. The resin was equilibrated with 10 ml buffer C. The flow rate was ~ 400 μ l per min throughout the chromatography. The hybridization supernatant was loaded onto the column. The flow through was reloaded 2 additional times. The column was rinsed with 17.5 ml buffer C followed by 7.5 ml buffer C containing 150 mM NaCl, and 5 ml of 0.75 $\times$ buffer C. Three fractions were eluted (~ 100, 300 and 250 μ l) with 0.75 $\times$ buffer H containing 50 mM freshly dissolved DTT. A final fraction (300 μ l) was collected with buffer H containing 150 mM NaCl and 50 mM DTT.

Sarkosyl gel assay

Hybridization conditions were optimized using a simple gel assay. The unmodified oligonucleotide was 5' kinase endlabeled and purified with a Bio Spin 6 column (Bio-Rad), deproteinized, and ethanol precipitated. After hybridization of the oligonucleotide to chromatin or DNA, the EDTA concentration was adjusted to 10 mM, and 1/10 volume of 10 $\times$ loading buffer (10 $\times$ TAE, 1.25% sarkosyl, 20% Ficoll, 0.1% bromphenol blue) was added. Up to 50 μ l of the hybridization mixture was loaded in the 2mm $\times$ 5mm $\times$ 8 mm wells of a 0.6% agarose gel containing 0.125% sarkosyl and TAE buffer. The gel was run at 1.25 V/cm for 16 h without being submerged in the TAE buffer (35). The gel was ethidium bromide stained and then either fixed in 7% TCA for 30 min and dried, or electrotransferred to a Zeta-Probe membrane and exposed to Kodak X-OMAT AR film.

Dot blots

Samples were deproteinized 3 h at 45°C by adding SDS to 0.5% and proteinase K to 0.5 mg/ml, extracted with phenol: chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), and then ethanol precipitated. The pellets were washed in 70% ethanol and resuspended in TE. DNA concentration was determined by scintillation counting. To each sample (< 100 ng DNA) 5 μ g of carrier tRNA was added, and the volume was adjusted to 500 μ l with 1 $\times$ SSPE. The nylon membrane was soaked in H₂O and clamped in a Schleicher & Schuell dot blot device. Each well was washed with 500 μ l 1 $\times$ SSPE, filled with the DNA at a slow flow rate, and rinsed

with 500 ml 1 $\times$ SSPE. The membrane was removed, and the DNA was fixed and denatured by putting the membrane DNA side up on top of three 3MM papers soaked in 0.4 M NaOH for 10 min. After 5 min a dry 3MM paper was put on top and removed when soaked with liquid. Finally the membrane was neutralized with 2 washes in 2 $\times$ SSPE. Prehybridization was done in 1.5 $\times$ SSPE, 0.5 mg/ml salmon sperm DNA, 1% SDS, and 0.5% Carnation non fat milk at 68°C for > 6 h. Hybridizations were done at the same conditions for > 24 h using heat denatured, [³²P]-labeled probes (random primer kit from Boehringer Mannheim). Post hybridization washes (15 min each) were at room temperature with 2 $\times$ SSC, 1 $\times$ SSC, 0.5 $\times$ SSC, 0.1 $\times$ SSC followed by a 30 min wash at 50°C with 0.1 $\times$ SSC. All wash buffers contained 0.1% SDS. Each dot blot experiment included at least duplicates of each sample, control dots containing the purified SUEHGR fragment from the plasmid pCO2A (36), and a dilution series of pCO2A.

Denaturing acrylamide gels and probing with strand specific probes

An 8 M urea 6% acrylamide gel (Maniatis *et al.*, 1982) was run in order to analyze the length of the 3' and 5' termini of the SUEHGR chromatin. The gel was electrotransferred to a Zeta Probe membrane, fixed, and prehybridized as described. Hybridization with the 5' kinase endlabeled oligonucleotide was done at 55°C in 1 M NaCl, 1% SDS, 50 μ g/ml salmon sperm DNA, 25 mM NaPO₄ pH 7.0, and 10% dextran sulfate for 24 h. The washes were as described for the dot blot except the last wash was at 40°C with 0.1 $\times$ SSC and 0.1% SDS. Stripping of the probe was achieved by incubation of the filter for 30 min in 0.4 M NaOH at 37°C followed by 2 washes in 2 $\times$ SSPE and prehybridization.

Quantitation of autoradiograms and ethidium bromide stained gels

Autoradiograms were digitized using a cooled charge coupled device (CCD) camera (Star 1, Photometrics). The images were converted to optical densities on a Silicon Graphics IRIS workstation. Integrated band intensities were calculated using the EMPRO image processing package. Quantitation of the dot blots was done similarly. The linearity of response was checked using the pCO2A dilution series. Ethidium bromide stained gels were destained in H₂O for several hours. Fluorescence was recorded with the Star 1 camera using a Mineralight[®] UV 254 nm lamp as light source. The illumination was at a ~45° angle. The CCD camera was equipped with an Wratten orange filter and shielded from direct UV light. Bands containing < 1 ng DNA were not visible by eye, but could be quantitated easily by 10 min exposures. Fluorescence intensities were analyzed with EMPRO after the dark current and local background were subtracted.

Micrococcal nuclease digestion of nuclei and purified SUEHGR

Nuclei were resuspended to ~50 μ g/ml in buffer A containing 1 mM CaCl₂, preincubated at 37°C for 3 min, and incubated with different amounts of micrococcal nuclease for 5 min. The digestion was stopped by adding EDTA (10 mM) and SDS (0.5%). For the DTT released fractions (~0.2 mg/ml) the total Ca⁺⁺ concentration was adjusted to 2 mM, 3 μ g tRNA were added, and preincubation and digestion done as for nuclei. The DNA was purified, electrophoresed in 2% agarose and electrotransferred to Zeta Probe. Hybridizations were performed as described.

Low salt chromatin spreads

Chromatin fractions were desalted on Sepharose 4B at 4°C. The elution buffer was 5 mM triethanolamine pH 7.0 and 0.2 mM EDTA. The void volume peak was fixed with 0.4% glutaraldehyde at 4°C for 12–18 h. Copper EM grids (400 mesh) were coated with a carbon film, put on top of a droplet containing ethidium bromide (30 µg/ml) for 15 min, and dried on filter paper. Spreading (37) was done by adding Benzyltrimethylammonium chloride (BAC) to the fixed chromatin (2.5 µg/ml). After 30 min on ice 7.5 µl aliquots were placed on parafilm, and the grids were put on top for 5 min. The grids were washed by floating them for 10 min on top of H₂O followed by a 3 sec rinse in Ethanol. The air dried grids were rotary shadowed with 80:20 Pt:Pd.

Electron microscopy and nucleosome counting

A Zeiss EM 902 was used for microscopy. The grids with the purified SUEHGR were searched for molecules. Each molecule that came into the field was photographed at

30,000× magnification. The beads on each molecule were counted 3 times from prints at 100,000× magnification.

RESULTS**Outline of the method**

The seven steps of nucleoprotein hybridization are schematically shown in Fig. 1.

1. Solubilization of gene size chromatin fragments by digestion of nuclei with restriction enzymes at low Mg⁺⁺ concentrations and EDTA lysis of the nuclei. 2. Generation of single-strand termini by T7 gene 6 exonuclease digestion. 3. Synthesis of an oligonucleotide complementary to the sequence adjacent to the restriction site on the targeted gene. 4. Biotinylation of the oligonucleotide. 5. Hybridization in solution between oligonucleotide and chromatin fragments. 6. Immobilization of the oligonucleotide/chromatin hybrids on an avidin matrix. 7. Elution of the purified chromatin fragments by DTT cleavage of the disulfide bond in the linker of the biotin analog. The salt and temperature conditions throughout the procedure are

Fig. 1. See text

compatible with intact chromatin. Figure 2 shows the pertinent restriction sites of the SUEHGR, the sequences of the synthetic oligonucleotides, and the hybridization probes used for filter hybridizations.

Solubilization of chromatin fragments with restriction enzymes

The chromatin was solubilized by digestion with restriction enzymes at low Mg^{++} concentrations (38). For the isolation of the whole SUEHGR repeat the single cutter HindIII was used. Figure 3 shows the result of such a digestion of sea urchin nuclei from different developmental stages. SUEHGR monomers (6.5 kb) and multimers are found in the insoluble (P) and soluble (SN) fractions. The use of dialysis instead of centrifugation (as used earlier, ref. 38) for the buffer changes reduced clumping of the fragile nuclei and increased the yield of solubilization (15–30%). Figure 3 shows that the yield was not limited by the efficiency of restriction. The chromatin in the pellet was cut with similar efficiency as the soluble chromatin. The active SUEHGR (10 h) was cut more frequently than the inactive gene (18 h, 36 h), but the solubilization efficiencies were similar for all stages of

Fig. 2. Map of the *S. purpuratus* early histone gene repeat. 1) Pertinent SUEHGR restriction sites and coding regions are indicated. 2) The sequences of the SalI 33^{mer} and the HindIII 17^{mer} oligonucleotides are complementary to the 3' strand upstream of the SalI and HindIII sites. 3) Hybridization probes. Fragments B are not complementary to the HindIII 17^{mer}. pCO2A contains one SUEHGR.

Fig. 3. Southern blot of chromatin fractions after HindIII digestion. Nuclei isolated from embryos at the indicated time points were digested with HindIII, lysed, and centrifuged at 10,000 g for 5 min. The DNA was purified from the supernatants (SN) and the pellets (P), and electrophoresed in 0.6% agarose. The Southern blot was probed with nick translated pCO2A. The weak band at 4 kb could be due to sequence polymorphism in the SUEHGR (39).

development (15–30%). Therefore entrapment in the nuclear debris seems to limit chromatin solubility. The HindIII solubilized chromatin was not enriched in SUEHGR sequences. On the other hand, SalI cut bulk chromatin less efficiently than the SUEHGR, enriching the SUEHGR 3–5 fold during solubilization.

Exonuclease digestion

Chromatin fragments with 3' single stranded ends were produced by digestion with λ exonuclease or T7 gene 6 exonuclease. The alternative approach of using ExoIII was not attempted, because this enzyme was reported to have some double strand nicking activity (40). Low monovalent salt conditions were chosen in order to avoid chromatin precipitation in the presence of 0.5 mM Mg^{++} . The proteins associated with the chromatin have an inhibitory effect on the exonucleases. After extensive digestion of the soluble chromatin, only a small fraction of the [³H]thymidine labeled DNA was TCA soluble (1–3% acid solubility after 1–3 h at 37°C).

Figure 4 shows that a λ exonuclease exposed intact 3' termini adjacent to the SalI site of SUEHGR chromatin. After restriction, exonuclease treatment, and deproteinization the DNA was cut with XhoI to obtain a small terminal fragment with a maximum size of 363 bp. The length of both terminal strands were determined with high resolution on a denaturing gel. The Southern blot of the gel was probed first with a single-stranded oligonucleotide complementary to the 3' upstream end of the SalI site (Fig. 4A) in order to measure the size of the non-exonucleated strand. The sharp bands in lanes 2 and 3 indicate that the exposed ends were intact. The same blot was stripped and then probed with double stranded probe A (Fig. 4B) in order to measure the length of both terminal strands. Quantitation of the autoradiograms revealed that > 80% of SUEHGR terminal fragments had single stranded ends and more than 50% of the molecules had 3' termini larger than 50 bp. The band on top

Fig. 4. Denaturing gel and strand-specific probing of λ exonuclease-treated chromatin. Chromatin was solubilized with SalI and digested with 5 U/ μ g λ exonuclease. The DNA was purified, digested with XhoI, electrophoresed in 8M urea, 6% acrylamide, and electrotransferred to Zeta Probe. (A) Initial probing with the single stranded oligonucleotide SalI 33mer. (B) The same filter stripped and re-probed with double stranded probe A (Fig. 2). M: partial digest of the SUEHGR as molecular weight markers. Lane 1 was incubated without exonuclease for 60 min. Lanes 2 and 3 were incubated with exonuclease 5 min and 60 min, respectively.

Fig. 5. Densitometer traces of sarkosyl gel autoradiogram. [32 P]-labeled oligonucleotide (HindIII 17^{mer}, 5 ng/ml) was hybridized in buffer H for 8 h with the following targets: a) pCO2A plasmid DNA; b) and c) chromatin; and d) DNA extracted from solubilized and exonuclease-treated chromatin. All targets were digested with HindIII and T7 gene 6. The chromatin was from 18 h nuclei. Procedures as described in Methods. The sodium chloride concentrations during hybridization are shown in the inset. All traces were normalized to a constant amount of loaded SUEHGR sequence (determined from dot blots). The integrated signal intensities over all bands are shown. The plasmid fragment had a higher mobility due to more extensive digestion with T7 gene 6.

of the gel represents XhoI-XhoI fragments derived from SUEHGR multimers. T7 gene 6 exonuclease produced similar termini adjacent to the HindIII site (not shown).

Chromatin oligonucleotide hybridization

High salt and temperature are known to maximize the rate and specificity of DNA hybridizations. Such conditions are not compatible with intact chromatin structure (41,42). However, we have previously shown that 100 mM salt and 37°C were stringent enough for specific hybridization to SV 40 minichromosomes (26). We developed a simple assay to determine the stringency and kinetics of hybridization of the oligonucleotide to SUEHGR chromatin.

Figure 5 shows that the SUEHGR chromatin and DNA were specifically hybridized to an oligonucleotide at 100 mM NaCl, 37°C. Exonuclease treated chromatin or DNA was hybridized with a 5' [32 P]-labeled oligonucleotide. The hybridization mixtures were electrophoresed on a gel containing enough sarkosyl for the chromatin to co-migrate with DNA of the same size (35). Clearly, the [32 P]-labeled oligonucleotide bound to DNA of the same size as the SUEHGR chromatin. The specificity was similar for pCO2A and for sea urchin chromatin (trace a vs. b) and for chromatin and genomic DNA (trace c vs. d). Therefore, the chromosomal proteins do not prevent hybridization. Hybridization was not completed after 8 h in 50 mM NaCl (trace c vs. b). The SalI 33^{mer} hybridized to SalI cut chromatin with similar efficiency and stringency (not shown).

Using this assay, the minimum amount of T7 gene 6 exonuclease required to achieve maximal hybridization efficiency was determined. A plateau value was reached at ~50 U/mg. This corresponds to an acid solubility of 2.8%. For standard preparative hybridizations 8 U T7 gene 6 exonuclease/mg DNA was added. This corresponds to a hybridization efficiency of ~70% of the maximum achieved with a limit exonuclease digestion.

Lambda exonuclease treated chromatin gives rise to a strong broad signal in the 4kb region of the sarkosyl gel and a specific hybridization signal that was only half that of T7 gene 6 treated

chromatin. Additional studies revealed that the λ exonuclease bound non-specifically to the oligonucleotide (not shown). This exonuclease is a highly processive enzyme and therefore binds strongly to oligonucleotides. The reduction in hybridization efficiency was probably due to the λ exonuclease remaining attached to the exposed single stranded tails. We therefore used T7 gene 6 exonuclease for all preparative isolations.

The kinetics of the reaction were studied with the sarkosyl assay and plotted according to a pseudo first order kinetics (26). The $t_{1/2}$ at a oligonucleotide concentration of 0.7 nM and a chromatin concentration of ~50 μ g/ml were 7 h at 100 mM NaCl and 10 h at 50 mM NaCl. The resulting hybridization rate constants were $1.8 \times 10^4 \text{ M}^{-1}\text{s}^{-1} \pm 0.09 \text{ M}^{-1}\text{s}^{-1}$ at 50 mM NaCl and $3.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1} \pm 0.23 \text{ M}^{-1}\text{s}^{-1}$ at 100 mM NaCl. These rates display the NaCl dependence expected for a hybridization reaction and are about an order of magnitude higher than the rates for hybridization of mercurated plasmid fragments to chromatin and to DNA (26).

Affinity chromatography

The chromatin oligonucleotide hybrids were separated from the bulk chromatin using an avidin affinity matrix. The biotin/avidin interaction ($K_{\text{diss}} = 10^{-15}$) provides a high binding efficiency. The use of 5' amino modified oligonucleotides allowed the synthesis and HPLC purification of a fully biotinylated oligonucleotide. Non denaturing elution was achieved by using a biotin analog that contained a cleavable disulfide bond in its linker (NHS-SS-Biotin, Pierce). The Vectrex Avidin D matrix bound 1ng of the biotinylated 17^{mer} per mg of dry matrix with ~70% efficiency. DTT elution was quantitative. The type of affinity matrix employed determined the extent of background binding. Background chromatin is the fraction of non-SUEHGR chromatin that binds to the affinity matrix and is released with DTT. Minimizing background is critical to achieve the maximum enrichment. Vectrex Avidin D was found to have low background binding properties (<0.009% of loaded [3 H] Thymidine counts in the peak DTT fraction). Addition of oxidized glutathione (GSSG) and IAcNH₂ to the hybridization and rinsing buffers, rinsing the column with 150 mM NaCl, and eluting the chromatin in 75 mM NaCl further decreased the background 3-fold.

Figure 6 shows a typical column elution profile. The SUEHGR content of the different fractions was determined by dot blot hybridization. The enrichment was calculated by dividing the hybridization signal per ng DNA for the enriched fraction by the signal for genomic DNA. The peak DTT fraction contained >800 fold enriched SUEHGR sequences. The purity can be calculated by multiplying the enrichment factor by the SUEHGR content in the genome. We determined the genomic SUEHGR content by comparing the hybridization signal of the genomic DNA to that of a plasmid fragment containing the SUEHGR. The SUEHGR was $0.15 \pm 0.05\%$ of the sea urchin genome, which is substantially lower than the 0.25–0.45% previously reported (43). More accurately, the purity was calculated by comparing the dot blot signal of the column fractions with the signal from similar amounts of the isolated pCO2A SUEHGR insert on the same filter. The purities determined in this fashion for the peak DTT fraction for four independent isolations were 83%, 89%, 105%, and 132%.

Figure 7 shows the result of an independent electrophoretic assay of the purity and molecular weight distribution of the isolated SUEHGR-DNA. The chromatin fractions were deproteinized and electrophoresed. The ethidium bromide fluorescence was recorded. Subsequently a Southern blot of the

Fig. 6. Vectrex affinity column elution profile of *S. purpuratus* chromatin after nucleoprotein hybridization. HindIII solubilized chromatin from 18 h nuclei was hybridized to the biotinylated HindIII 17^{mer} for 24 h. Affinity chromatography was performed as described. F.T. is the flow through fraction. NaCl concentrations of the column buffers are indicated. a) Chromatin concentrations were determined by [³H] scintillation counting. b) The SUEHGR concentration was calculated by multiplying the chromatin concentration by the purity. Conversion from enrichment (determined on dot blots) to purity was made using the results from the gel analysis of the D2 fraction, which showed that an enrichment of 840× corresponded to 100% purity (Fig. 7 below). c) The enrichment was determined by comparing the dot blot hybridization signals of the column fractions to those of nuclear DNA with probe B. d) The yield of the column procedure is the enrichment multiplied by the amount of chromatin in the fraction compared to the amount loaded onto the column.

same gel was probed with probe B to determine the distribution of the SUEHGR sequences. The DNA size distribution of the solubilized chromatin and the purified SUEHGR are shown in Fig. 7 trace a and b, respectively. The SUEHGR distribution is shown in the Southern blot (trace c). Trace b and c are superimposable. Thus, the fluorescence between the dimer to pentamer bands was not due to background but due to overlapping SUEHGR bands. Quantitation of the peak and background signal in the ethidium bromide stained gel and autoradiogram showed that the purity of two independent preparations were 108% and 80%. Comparison of the molecular weight distribution on Southern blots of purified SUEHGR and unpurified soluble SUEHGR chromatin show that the SUEHGR chromatin has been purified as integral multiples of 6.5 kb without any degradation of the DNA (not shown).

To verify that the enrichment process is due to hybridization of chromatin to the oligonucleotide and not due to some other process, a negative control was performed. HindIII-solubilized chromatin without exonuclease treatment was used in the hybridization reaction with the 17^{mer} oligonucleotide. No hybridization was detectable by the sarkosyl gel assay. Affinity chromatography of this mock hybridization mixture gave a small (< 50 fold) but reproducible enrichment (not shown). We interpret this result as a partial strand invasion that produces hybrids that are stable under the affinity column conditions. We conclude, however, that the much more significant enrichment seen by nucleoprotein hybridization was due to sequence-specific hybridization.

Fig. 7. Determination of SUEHGR purity by agarose gel analysis of DNA from DTT-eluted chromatin. Nucleoprotein hybridization was performed as described in the methods using HindIII solubilized chromatin. The DNA from different column fractions was purified and electrophoresed in 0.6% agarose. One dimensional projections of fluorescence intensities and autoradiographic densities are shown. The traces were scaled to give the same peak height for the strongest band. a) Ethidium bromide fluorescence of the chromatin after hybridization (loading fraction). b) Ethidium bromide fluorescence of the DTT eluted chromatin (isolated SUEHGR). c) Southern blot of lane b hybridized with probe B.

Fig. 8. Micrococcal nuclease digestion of isolated SUEHGR. SUEHGR chromatin was purified from nuclei isolated at the indicated times of embryogenesis and digested with different amounts of micrococcal nuclease. Nuclei of the same batches were also digested. The DNA was purified and electrophoresed in 2% agarose. The nuclear lanes (lanes 1,4,9) contained ~5 mg and the isolated SUEHGR lanes (2,3,5,6,10) contained < 50 ng total DNA. Lanes 7,8 contained DTT released material from negative controls where the chromatin target was not exonucleated. These lanes showed a weak pattern similar to lanes 5 and 6 after overexposure of the filter. A) Autoradiogram of the Southern blot probed with pCO2A, containing one SUEHGR. B) Scaled densitometer traces of the indicated lanes of the same autoradiogram. N., nuclei; i.S., isolated SUEHGR.

Characterization of the isolated SUEHGR by micrococcal nuclease digestion

Micrococcal nuclease is an established probe for the investigation of the SUEHGR chromatin structure (44, 45). To assay if the main structural features were maintained during the purification, isolated SUEHGR chromatin and nuclei from different developmental stages were digested with micrococcal nuclease followed by Southern hybridization with SUEHGR sequences. Figure 8 shows the results of this assay. The isolated SUEHGR (lanes 5,6,10) show clear nucleosomal ladders for the inactive

Fig. 9. Electron micrographs of BAC spread purified SUEHGR. Isolated SUEHGR from 18 h nuclei were desalted on a Sepharose 4B column and BAC spread with the microdroplet method. The grids were rotary shadowed with Pt:Pd. The bar is 0.2 μ m. A) Micrographs a,b,c and d show SUEHGR monomers. Micrograph e shows a dimer molecule. B) Histogram of the number of beads on each molecule. Three molecules for which the standard deviation of 3 countings was >10% of the mean were not included. Six molecules had more than 100 beads.

chromatin (18 h and 36 h) that are virtually identical to those of intact nuclei (lanes 4,9). The isolated 10 h SUEHGR (lanes 2,3) have a diffuse pattern characteristic for the active SUEHGR (lane 1, and ref. 44,45). This pattern indicative of non-regularly spaced nucleosomes is also preserved during the isolation process. These results clearly establish that the purified material is chromatin with the same main structural features as in the nuclei. Significant nucleosome loss or sliding did not occur during isolation.

EM of the isolated material

The isolated SUEHGR is estimated to have a molecular weight of $\sim 9 \times 10^6$ D/monomer. Electron microscopy is particularly suited to give a low resolution picture of molecules in this size range. Electron microscopy also allows the study of individual molecules, which enables an assessment of the homogeneity of the isolated SUEHGR. Such information is not obtainable by biochemical techniques. Figure 9A shows a low salt BAC spread of the DTT eluted material originating from 18 h nuclei. The molecules have the expected beads-on-a-string structure typical of inactive chromatin. The number of beads on each of 45

molecules were counted. The result of this analysis is given in Fig. 9B. In this highly pure preparation (> 90%) the SUEHGR monomers appear homogeneous and contain 27 ± 3 beads. This is in close agreement with 29.5 nucleosomes calculated using the repeat length of SUEHGR in nuclei (45). The counting of beads on higher oligomers was less reliable, because incomplete spreading led to strand overlap. These micrographs confirm the results of micrococcal nuclease digestion, and show the feasibility of using EM for a comparative study of active and inactive genes.

Histone integrity and histone exchange

In order to verify that no proteolysis occurs during nucleoprotein hybridization, aliquots were removed throughout the procedure, analyzed by SDS-PAGE, and the histone composition was compared to nuclei. Proteolysis of the histone proteins was completely prevented by leupeptin. The four core histones as well as H1 are quantitatively preserved throughout the multiple dialysis, digestions, dilutions and affinity chromatography steps (not shown). Unfortunately, the electrophoretic pattern of the purified SUEHGR chromatin could not be determined because of insufficient material for conventional visualization by Coomassie blue staining or autoradiography of [^{35}S] labeled proteins.

The conditions employed during nucleoprotein hybridization were designed to preserve the DNA protein interactions and minimize protein exchange. The results obtained with SV 40 justified these conditions (26). In the following experiment we tried to address the question of histone exchange between bulk chromatin and SUEHGR. [^3H]Thymidine labeled, exonuclease treated chromatin was mixed with [^{35}S]methionine labeled, non-exonuclease treated chromatin. After hybridization to the 17^{mer} oligonucleotide for 24 h at 37°C the amount of [^{35}S]-labeled protein in the DTT eluted fraction was determined. Only a small percentage of the loaded radioactivity was eluted from the columns with DTT ($0.025 \pm 0.004\%$ from the hybridizable sample and $0.026 \pm 0.003\%$ from a non-hybridizable control where the chromatin had not been exonuclease treated). Thus most of the labeled protein eluted was not bound to the hybrids. The different chromatin fractions were ethanol precipitated and the proteins were analyzed by gel electrophoresis. Each of the five histone bands was excised, H_2O_2 hydrolyzed, and counted by liquid scintillation (46). The ratio, R , of ^{35}S counts recovered in the histone bands to the amount of [^3H]Thymidine used for the ethanol precipitation of each sample was determined for the different chromatin fractions. Complete exchange would produce the same labeled histone to labeled DNA ratio in the DTT fraction as in the flow through ($R_{\text{DTT}} = R_{\text{flow through}}$). In the case of 100% purity and absence of exchange an R value = 0 is expected. The presence of background chromatin (~20% as quantitated by dot blots) leads to a practical minimum value of $R = 0.2 R_{\text{flow through}}$. Comparing the R values of enriched and flow through fractions of two independent experiments gave the following estimates of core histone exchange: Exp #1, $32\% \pm 24\%$; Exp #2, $17\% \pm 16\%$. The standard deviations refer only to the statistical uncertainty of the scintillation counting. It was not possible to measure H1 exchange using this procedure due to the small number of counts in the H1 band.

The results are of low accuracy because of the inherently low specific activity of the radioactively labeled histones, but they are consistent with minimal core histone exchange as measured previously (26). The EM and micrococcal nuclease results argue strongly for retention of a large majority of the original proteins (see Discussion).

DISCUSSION

The technique

The results presented here clearly show that nucleoprotein hybridization can be used to purify the sea urchin early histone genes as chromatin. Independent assays were developed for the exonuclease and the hybridization reactions to facilitate their optimization. The fact that efficient nucleoprotein hybridization has now been achieved at all four sites tested (2 in SUEHGR and 2 in SV 40) makes us confident that the technique is of general use for the enrichment of eukaryotic genes as chromatin. The most significant limitation seems to be the limited number of genes that occur in sufficient abundance (i.e. > 0.1% of the genome) to isolate biochemical quantities at high purity.

The digestion of nuclei with restriction enzymes produced soluble chromatin fragments with discrete lengths (Fig. 3) containing a full complement of histone proteins. Traditional protocols for the restriction of chromatin have used high Mg^{+2} concentrations (e.g., 30, 47, 48), which cause chromatin to aggregate, making cleavage and solubilization difficult. Protocols using low Mg^{+2} concentrations (0.5 mM) produce higher accessibility and solubility (38), and have general applicability to solubilization of intact, long chromatin. Only a fraction of the cleaved chromatin molecules became soluble (Fig. 3), which we attribute to entrapment of the genes on or within the nuclear debris. Recent progress in understanding the nuclear disassembly process (49,50) might eventually lead to more efficient solubilization.

The extent of exonuclease digestion of soluble chromatin was limited by the chromatin structure. A limiting value of ~3% TCA solubility was reached under standard incubation conditions, similar to earlier results with SV 40 (26), chicken, and sea urchin chromatin (not shown). The single-strand gel assay showed that both the SalI and HindIII 5' termini are efficiently digested, leaving >80% of the SUEHGR with intact 3' overhangs of an average size of ~50 bp (Fig. 4).

The use of the non-porous avidin D matrix resulted in low levels of background chromatin. The enrichments obtained using identical column conditions were similar for chromatin from different developmental stages. Adding GSSG and IACNH₂ to the hybridization buffer and GSSG and 150 mM NaCl to the column rinsing buffer reduced the background further so that chromatin from 18 h embryos could be enriched >700 fold in a single affinity step. The eluted material was >80% pure and contained chromatin pieces of discrete length (Fig. 7). Micrococcal nuclease digestion (Fig 8) assay revealed no indication of significant nucleosome rearrangement or subfractionation during nucleoprotein hybridization.

Because there are about 500 copies of the SUEHGR in the genome it is important to ask whether all copies have the same structure. Minor sequence variations in the SUEHGR have been described (39), however our Southern blots consistently show little variation in the length of the sequence repeat (Fig. 3 and 7). Heterogeneity in chromatin structure is much more difficult to detect, and would be detrimental to the use of nucleoprotein hybridization to study the proteins associated with active or inactive genes. Heterogeneity in multi-copy genes has been demonstrated in the case of rRNA genes, which exhibit micrococcal nuclease patterns and psoralen crosslinking results consistent with about equal amounts of active and inactive copies of the gene (51). Earlier studies of the SUEHGR of *P. miliaris* concluded that all of the copies of the genes (both at early blastula

and hatching blastula) had a very homogeneous chromatin structure, as assayed by nuclease hypersensitivity and protection (52).

The micrococcal nuclease digestion patterns of 18 and 36 h SUEHGR (Fig. 8) show a very clear pattern with very little background beneath the ladder. There is for example no band at ~290 bp which is a predominant feature of the active chromatin. In addition, it has been shown that DNaseI hypersensitive sites disappear completely after gene inactivation (45). The inactive, purified SUEHGR chromatin also seemed to be structurally homogeneous as indicated by the narrow distribution of the number of beads per SUEHGR monomer (Fig. 9).

The homogeneity of the active genes is less clear, because the only data comes from the micrococcal nuclease digests (Fig. 8). Clearly, the major fraction of 10 h SUEHGR chromatin is different from the 18 h chromatin (Lane 1 and 2 vs. 4 and 5). However, there might be some fraction that behaves like inactive chromatin. The weak but sharp bands seen at higher molecular weight (lane 2) could be due to a minor fraction of SUEHGR chromatin resembling the inactive chromatin. Other features of the gel are ambiguous, e.g. the band observed at ~440 bp (best visible in lane 3) could represent a dimer with the 220 bp repeat of inactive chromatin or be a compact trimer with a ~146 bp repeat of active chromatin. The data presented are insufficient to make a quantitative assessment of the structural heterogeneity. Because only a minor fraction of the 10 h genes seem to have an inactive structure the biochemical and structural studies of the isolated SUEHGR chromatin should not be seriously hindered.

Control experiments have shown that loss or exchange of core histones is minimal. Preservation of the original core histones is also indicated by the micrococcal nuclease digestion patterns (Fig. 8) and the EM analysis (Fig. 9). The EM analysis revealed that the SUEHGR monomers had 27 ± 3 nucleosomes. Because the average repeat length of SUEHGR in nuclei is 220 bp (44), we expected 29.5 nucleosomes per SUEHGR monomer. This slight discrepancy could be due to inherent gaps in the nucleosomal repeat (which would not affect the digestion ladder), to the inherent difficulties in counting nucleosomes by EM, or by an unexpected loss of <10% of the nucleosomes. The experiments presented measure only exchange that occurs during the prolonged incubations after the exonuclease steps, and were limited by the low specific activity of the proteins. Earlier experiments using SV 40 (26) assayed exchange throughout the isolation procedure and found less than 10% loss or exchange of the core histones.

We were not surprised by the lack of significant loss or exchange because previous studies have repeatedly found that core histones do not exchange at ~100 mM monovalent salt. Germond *et al.* (53) reported negligible loss or exchange of histones from SV 40 minichromosomes during 30 min incubations at 37°C with an equal amount of 100 mg/ml SV 40 form I DNA, as long as the salt concentration was less than 700 mM NaCl. Crémisi *et al.* (54) were unable to detect protein exchange after 2 h incubations of minichromosomes with DNA. Ilyn *et al.* (55) found little exchange of histone and non histone proteins after 24 h incubations of sheared chromatin with exogenous tRNA, DNA and other chromatin at 120 mM NaCl. Even nucleosome sliding was not detectable at 100–150 mM NaCl (54,56).

The presence of H1 on the purified SUEHGR was indicated by the preservation of the native micrococcal nuclease repeat length (Fig. 8). We didn't observe compact dimers, which have

been consistently observed by other investigators if H1 stripped chromatin is incubated at 37°C under physiological salt conditions (57,58). On the other hand H1 exchange on bulk chromatin is expected (59) during isolation of the nuclei, enzymatic digestions and nucleoprotein hybridization. We were unable to determine whether histone H1 exchange onto the SUEHGR took place.

Comparison with alternative methods

This general approach towards the task of purifying a chromatin fragment with a specific sequence has enabled the first purification of an RNA polymerase II gene as chromatin. Nucleoprotein hybridization can be used to isolate the same chromatin fragment in different functional states. This makes possible direct comparative studies between active and inactive chromatin fragments of the same DNA sequence.

This technique is superior in several aspects to the use of immunoprecipitation (e.g. 21–23), differential solubility (e.g. 11–20) or DNase I hypersensitivity (60), which have been used to enrich for certain subpopulations of chromatin. Those techniques cannot target a specific gene, because enrichment depends on physical properties or protein composition rather than DNA sequence. In addition the isolated chromatin is small and heterogeneous due to the use of non-specific enzymes.

Gene-targeted chromatin fractionations have been successful in cases where the physical properties of a large fraction of the genome were different from the bulk (e.g. satellite DNA, ref. 61; rDNA, ref. 25,30,47). These procedures are not based on any principle applicable to other genes. Partial protein analysis using conventional detection systems was possible in those cases due to the highly repetitive nature of these genes.

A more general approach that is also applicable to the isolation of biochemical quantities of a single copy gene has been the use of yeast minichromosomes (62). In principle any gene could be cloned into a yeast plasmid, its chromatin assembled *in vivo*, and subsequently reisolated. Unfortunately, the characterization of co-purified proteins has been difficult due to unspecifically bound proteins. This system is also of limited value for non-yeast genes for which the proteins and protein modifications available for the reconstitution will be different from those in their native environment.

Limitations and applications

At present it is reasonable to use nucleoprotein hybridization to purify eukaryotic genes representing >0.1% of the genome. Less abundant genes cannot be purified to homogeneity by single-step biotin-affinity chromatography. Indications are that the limiting factor is the binding of extraneous chromatin to the Avidin D columns rather than low stringency of hybridization. For higher eukaryotes, the current enrichment limits applications to multi-copy genes. For heterogeneous multi-copy genes, such as the rRNA genes from Friend cells, additional purification steps might be necessary to separate genes of different structure. Practical limitations in the number of cells and the amount of enzymes will restrict the yield to a few nanograms of specific genes, requiring micro-analytical analysis of the material by radiography of proteins radioiodinated to high specific activity and by EM of the isolated molecules.

The method as presented can be used for the study of SUEHGR-specific core histone proteins. Two dimensional gel electrophoresis will be used to investigate changes in sequence variants and covalent modifications of these proteins during sea urchin development. Because histones are the most abundant

proteins, they should be easily detectable on autoradiograms of gels containing radioiodinated SUEHGR proteins. Tyrosine labeling of histone proteins should give 10^4 times higher specific activity than what was achieved *in vivo* with [35 S]methionine, and should also allow the detection of proteins occurring in a single copy per SUEHGR. Radioiodination will also allow more detailed studies of protein exchange and loss.

Additional improvements in the method might be necessary to study some of the non-histone proteins and H1. Size exclusion chromatography might be required to separate SUEHGR-associated high molecular weight proteins from background proteins. Isolation of H1 or non-histone proteins with a low binding constant to their native chromatin fiber might require the use of lower salt concentrations or reversible fixation (63) to avoid loss or exchange.

We have demonstrated the feasibility of investigating the isolated material by EM (Fig. 9). Inter-SUEHGR homogeneity and intra-SUEHGR heterogeneity of nucleosome positioning will be studied by EM. Immunostaining techniques could also be used to map proteins along single SUEHGR molecules (64,65). If a heavy atom label can be put onto the hybridizing oligonucleotide, specific genes could be identified from micrographs of only partially-purified genes. Tagged and hybridized oligonucleotides have been identified previously in the EM (66). In addition, the amounts of material needed for EM are small (< 1 ng). Therefore partial purification by nucleoprotein hybridization, combined with gene-specific tagging might make EM studies of single copy genes possible.

ACKNOWLEDGMENTS

We are grateful to Dr. Shawn P. Williams and Michel F. Smith for setting up the image processing system. We also are grateful to Dr. Susan Klein and Karen Findling for their early contributions to this project. This work was supported by grants NIH GM 27937, NSF PCM-8317039, NSF DCB-9007295, and University of Michigan Office of the Vice President for Research. C.V. was supported by a Rackham Predoctoral Fellowship.

REFERENCES

- Rhodes, D. and Laskey, R.A. (1989) In Wassarman, P.M., Kornberg, R.D. (eds.), *Methods in Enzymology*, Academic Press, Vol. 170, pp. 575–585.
- Stein, A. (1989) In Wassarman, P.M., Kornberg, R.D. (eds.), *Methods in Enzymology*, Academic Press, Vol. 170, pp. 585–603.
- Shimamura, A., Jesse, B. and Worcel, A. (1989) In Wassarman, P.M., Kornberg, R.D. (eds.), *Methods in Enzymology*, Academic Press, Vol. 170, pp. 603–612.
- Sealy, L., Burgess, R.R., Cotten, M. and Chalkley, R. (1989) In Wassarman, P.M., Kornberg, R.D. (eds.), *Methods in Enzymology*, Academic Press, Vol. 170, pp. 612–630.
- Neubauer, B. and Hörz, W. (1989) In Wassarman, P.M., Kornberg, R.D. (eds.), *Methods in Enzymology*, Academic Press, Vol. 170, pp. 630–644.
- Van Holde, K.E. (1989) *Chromatin*. Springer Series in Molecular Biology. Springer-Verlag.
- Levinger, L., Barsoum, J. and Varshavsky, A. (1981) *J. Mol. Biol.*, **146**, 287–304.
- Levinger, L. and Varshavsky, A. (1982) *Cell*, **28**, 375–385.
- Huang, S.-Y. and Garrard, W.T. (1989) In Wassarman, P.M., Kornberg, R.D. (eds.), *Methods in Enzymology*, Academic Press, Vol. 170, pp. 116–142.
- Mirzabekov, A.D., Bavykin, S.G., Belyavsky, A.V., Karpov, V.L., Preobrazhenskaya, O.V., Shick, V.V. and Ebralidse, K.K. (1989) In Wassarman, P.M., Kornberg, R.D. (eds.), *Methods in Enzymology*, Academic Press, Vol. 170, pp. 386–408.
- Bonner, J., Gottesfeld, J., Garrard, W., Billing, R. and Uphouse, L. (1975) In O'Malley, B.D., Hardman, J.G. (eds.), *Methods in Enzymology*, Academic Press, Vol. 40, pp. 97–102.
- Bloom, K.S. and Anderson, J.N. (1978) *Cell*, **15**, 141–150.
- Ridsdale, J.A. and Davie, J.R. (1987) *Nucleic Acids Res.*, **15**, 1081–1096.
- Strätling, W.H. and Dölle, A. (1986) *Biochemistry*, **25**, 495–502.
- Strätling, W.H. (1987) *Biochemistry*, **26**, 7893–7899.
- Hanks, S.K. and Riggs, M.G. (1986) *Biochim. Biophys. Acta*, **867**, 124–134.
- Davis, A.H., Reudelhuber, T.L. and Garrard, W.T. (1983) *J. Mol. Biol.*, **167**, 133–155.
- Kumar, S. and Leffak, M. (1986) *Biochemistry*, **25**, 2055–2060.
- Rose, S.M. and Garrard, W.T. (1984) *J. Biol. Chem.*, **259**, 8534–8544.
- Razin, S.V., Yarovaya, O.V. and Georgiev, G.P. (1985) *Nucleic Acids Res.*, **13**, 7427–7444.
- Dorbic, T. and Wittig, B. (1986) *Nucleic Acids Res.*, **14**, 3363–3376.
- Dorbic, T. and Wittig, B. (1987) *EMBO J.*, **6**, 2393–2399.
- Hebbes, T.R., Thorne, A.W. and Crane-Robinson, C. (1988) *EMBO J.*, **7**, 1395–1402.
- Allegra, P., Sterner, R., Clayton, D.F. and Allfrey, V.G. (1987) *J. Mol. Biol.*, **196**, 379–388.
- Amero, S.A., Ogle, R.C., Keating, J.L., Montoya, V.L., Murdoch, W.L. and Grainger, R.M. (1988) *J. Biol. Chem.*, **263**, 10725–10733.
- Workman, J.L. and Langmore, J.P. (1985) *Biochemistry*, **24**, 7486–7497.
- Kedes, L.H. (1979) In Shell, E.E. (ed.), *Ann. Rev. Biochem.*, Vol. 48, pp. 837–870.
- Weinberg, E.S., Hendricks, M.B., Hemminki, K., Kuwabara, P.E. and Farrelly, L.A. (1983) *Developmental Biology*, **98**, 117–129.
- Jones, R.W. (1978) *Biochem. J.*, **173**, 155–164.
- Prior, C.P., Cantor, C.R., Johnson, E.M., Littau, V.C. and Allfrey V.G. (1983) *Cell*, **34**, 1033–1042.
- Amero, S.A., Montoya V.L., Murdoch, W.L., Ogle, R.C., Keating, J.L. and Grainger, R.M. (1988) *J. Biol. Chem.*, **263**, 10734–10744.
- Osheim, Y.N., Miller, Jr., O.L., Beyer, A.L., (1985) *Cell*, **43**, 143–151.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor University Press, Cold Spring Harbor.
- Weidman, P.J. and Kay, E.S. (1986) In Schroeder, T.E. (ed.) *Methods in Cell Biology*, Academic Press, Vol. 27, p.121.
- Ackerman, S., Bunick, D., Zandomeni, R. and Weinmann, R. (1983) *Nucleic Acids Res.*, **11**, 6041–6064.
- Overton, G.C. and Weinberg, E.S. (1978) *Cell*, **14**, 247–257.
- Thoma, F., Koller, Th. and Klug A. (1979) *Cell Biology*, **83**, 403–427.
- Workman, J.L. and Langmore, J.P. (1985) *Biochemistry*, **24**, 4731–4738.
- Yager, L.N., Kaumeyer, J.F., Lee, I. and Weinberg, E.S. (1987) *J. Mol. Evol.*, **24**, 346–356.
- Riley, D. and Weintraub, H. (1978) *Cell*, **13**, 281–293.
- Kinoshita, S. (1976) *Exp. Cell Res.*, **102**, 153–161.
- Ausio, J., Sasi, R. and Fasman, G.D. (1986) *Biochemistry*, **25**, 1981–1988.
- Weinberg, E.S., Overton, G.C., Shutt, R.H. and Reeder, R.H. (1975) *Proc. Nat. Acad. Sci. USA*, **72**, 4815–4819.
- Wu, T.-C. and Simpson, R.T., (1985) *Nucleic Acids Res.*, **13**, 6185–6203.
- Fronk, J., Tank, G. A. & Langmore, J.P. (1990) *Nucleic Acids Res.*, **18**, 5255–5263.
- Tishler, P.V. and Epstein, C.J. (1968) *Anal. Biochem.*, **22**, 89–98.
- Reynolds, W.F., Bloomer, L.S. and Gottesfeld, J.M. (1983) *Nucleic Acids Res.*, **11**, 57–75.
- McGhee, D.J., Wood, W.I., Dolan, M., Engel, J.D. and Felsenfeld, G. (1981) *Cell*, **27**, 45–55.
- Peter, M., Nakagawa, J., Dorée, Labbé, J.C., Nigg, E.A. (1990) *Cell*, **61**, 591–602.
- Ward, G.E. and Kirschner, M.W. (1990) *Cell*, **61**, 561–567.
- Conconi, A., Widmer, R.M., Koller, T. and Sogo, J.M. (1989) *Cell*, **57**, 753–761.
- Bryan, P. N., Olah, J. and Birnstiel, M.L. (1983) *Cell*, **33**, 843–848.
- Germond, J.E., Bellard, M., Oudet, P. and Chambon, P. (1976) *Nucleic Acids Res.*, **3**, 3173–3193.
- Crémisi, C., Pignatti, P.F. and Yaniv, M. (1976) *Biochem. Biophys. Res. Commun.*, **73**, 548–554.
- Ilyin, Y.V., Varshavsky, A. Y., Mickelsaar, U.N. and Georgiev, G.P. (1971) *Eur. J. Biochem.*, **22**, 235–245.
- Glotov, B.O., Rudin, A.V. and Severin, E.S. (1982) *Biochim. Biophys. Acta*, **696**, 275–284.
- Spadafora, C., Oudet, P. and Chambon, P. (1979) *Eur. J. Biochem.*, **100**, 225–235.
- Watkins, J.F. and Smerdon, M.J. (1985) *Biochemistry*, **24**, 7279–7287.

59. Caron, F. and Thomas, J.O. (1981) *J. Mol. Biol.*, **146**, 513–537.
60. Dawson, B.A., Herman, T. and Lough, J. (1989) *J. Biol. Chem.*, **264**, 12830–12837.
61. Zhang, X.Y. and Hörz, W. (1982) *Nucleic Acids Res.*, **10**, 1481–1494.
62. Dean, A., Pederson, D.S. and Simpson, R.T. (1989) In Wassarman, P.M., Kornberg, R.D (eds.), *Methods in Enzymology*, Academic Press, Vol. 170, pp. 26–41.
63. Solomon, M.J. and Varshawski, A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6470–6474.
64. Harper, F., Florentin, Y., and Puvion, E. (1984) *EMBO J.*, **3**, 1235–1241.
65. Bustin, M., Goldblatt, D. and Sperling, R. (1976) *Cell*, **7**, 297–304.
66. Brantley, J.D. and Beer, M. (1989) *Gene Anal. Techn.*, **6**, 75–78.