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Nucleoprotein Hybridization: A Method for Isolating Specific Genes as High Molecular Weight Chromatin†

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ABSTRACT: We describe a new technique designed to isolate specific eukaryotic genes as native oligonucleosome fragments. The isolation method consists of hybridization of single-stranded termini of chromatin restriction fragments to complementary mercurated DNA probes, followed by isolation of the hybrids by sulfhydryl-Sepharose chromatography. SV40 minichromosomes were used to test the effectiveness of the technique. About 80% of *Kpn*I- or *Bam*HI-restricted and λ exonuclease treated SV40 minichromosomes hybridized to an appropriate DNA probe after a 12-h hybridization reaction under mild conditions (0.1 M aqueous salt, 37 °C, pH 8). When the restricted minichromosomes were mixed with a 15-fold excess of "background" chromatin from sea urchin embryos, nucleoprotein hybridization was able to reisolate the SV40 chromatin to 88% purity with a 63% yield. This represented a 115-fold enrichment of specific genes as chromatin. Results of electron microscopy and polyacrylamide gel electrophoresis indicate that the hybridized SV40 chromatin has not lost the major chromosomal proteins characteristic of SV40 nor acquired significant amounts of protein due to exchange with background chromatin. Our experimental results show that it is currently possible to isolate repeated genes from higher eukaryotes for structural and biochemical study of the proteins involved with gene regulation.

While very significant progress has been made in the understanding of gene structure at the level of the nucleic acid sequence, little is known about the ways in which histone and non-histone chromosomal proteins interact with DNA sequences to control transcription. Largely, our ignorance about the control of transcription is the result of the inability to isolate and study specific sequences of DNA still bound to the protein and RNA molecules that are responsible for the structure and function of those sequences in vivo.

Currently, the only way to identify the regulatory proteins and to elucidate their molecular functions is to study the transcription of reconstituted genes in vitro using purified components or in vivo using the amphibian oocyte system [reviewed by Manley (1983), Wickens & Laskey (1981), and Gurdon & Melton (1982)]. To isolate putative regulatory proteins from a complex mixture of chromosomal proteins, DNA affinity techniques have been used to collect proteins that bind in vitro to regulatory DNA sequences [e.g., see Weideli et al. (1980) and Emerson & Felsenfeld (1984)]. This approach is undoubtedly very useful yet is susceptible to several potential artifacts due to the complexity of eukaryotic chromosomal proteins and to the likelihood that regulation in vivo involves the simultaneous or sequential binding of several proteins on a gene [reviewed by Brown (1984)].

There is a clearly expressed need [e.g., see Wickens & Laskey (1981) and Manley (1983)] to isolate individual genes as intact nucleoprotein molecules in the amounts necessary for biochemical and in vitro transcription studies, in order to complement and validate the gene reconstitution studies mentioned previously. Thus far, the attempts to isolate specific genes as chromatin have been limited to three types of chromatin, namely, satellite, ribosomal RNA, and 5S RNA genes (Zhang & Horz, 1982; Prior et al., 1983; Reynolds et al., 1983). These partial purifications were possible due to the special physical characteristics of the chromatin involved. Unfortunately, the strategies used for isolation of satellite, ribosomal, and 5S chromatin cannot be applied easily to other

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genetic elements. General methods of isolation of specific pieces of the genome as chromatin are clearly desirable.

The base sequences that confer individuality on genes as DNA are the ultimate key to a general method of isolating genes as chromatin, since the base sequence is the *only* property known to be unique to a specific fragment of the chromosome. Quite surprisingly, there have been no reports of using nucleic acid hybridization in order to label or isolate specific segments of nucleoprotein.

We have found that routine methods of nucleic acid enzymology and nucleic acid hybridization can be used to directly isolate unique sequence, high molecular weight, chromatin fibers. By employing moderate temperatures and ionic conditions throughout the isolation procedures, we have minimized loss and exchange of gene-specific chromosomal proteins. Since our approach makes no assumptions about protein composition or activity of the chromosome fibers, it could be used to isolate specific genetic elements at different stages of the cell cycle, at different stages of development, in different tissues, and in different organisms. Biochemical and structural studies of these purified chromatin fragments could address important questions about gene regulation.

In this paper, we describe the nucleoprotein hybridization procedure and its application to a model chromatin fragment, the "minichromosome" of the simian virus, SV40. In particular, we have investigated the kinetics and the specificity of the nucleoprotein hybridization reaction, as well as documented the fact that core histones are not lost or exchanged during the isolation procedure. A preliminary description of this work has appeared earlier (Workman & Langmore, 1984).

Description of the Method. Our approach to isolate specific genes as chromatin is based upon hybridization of a mercurated single-stranded DNA "probe" to the complementary sequence at the end of the "target" chromatin fragment followed by sulfhydryl-Sepharose chromatography. The general procedure is illustrated in Figure 1. To prepare the probe DNA, a fragment of the genome containing a part of the specific gene of interest is cloned into a bacterial plasmid, which is then cut with a restriction endonuclease. This DNA probe is then covalently bound to mercury and partially digested with exonuclease III in order to expose a few hundred bases at the 5′ ends. To prepare the chromatin for hybridization, nuclei are isolated from the cells of interest and restricted with the same restriction enzyme that was used to cleave the plasmid described previously. The soluble restricted chromatin is then prefractionated by sedimentation velocity on a glycerol gradient. Only those gradient fractions that are highly enriched in the target chromatin fragment are used for later steps. λ exonuclease is then used to expose 200–400 bases at the 3′ termini on the prefractionated chromatin molecules. Among these chromatin molecules will be a few (the number depending on the sequence complexity and efficiency of restriction and prefractionation) of the desired target chromatin molecules. By virtue of having the same sequences adjacent to the restriction site in the probe, these target molecules will have 3′ tails complementary to the 5′ tails of the probe. Incubation of the chromatin fragments with an excess of mercurated probe DNA for several hours at 37 °C in 0.11 M monovalent salt produces specific hybrids between the target nucleoprotein and the probe DNA. The probe and the hybrids are then specifically immobilized on a thiolated Sepharose column and subsequently eluted with a mercaptan-containing buffer.

Although nuclei acid hybridization is a well-tested method for isolation of specific-sequence RNA and DNA, it is not obviously appropriate for isolation of nucleoproteins. A review of the thermodynamic and kinetic evidence, however, fails to reveal any substantial reasons that single-stranded DNA on chromatin might have difficulty hybridizing to complementary single-stranded DNA. In general, renaturation of DNA is reasonably independent of strand mobility and limited in rate by the addition of 1 base pair to a preequilibrium intermediate or 2 or 3 base pairs [see Bloomfield et al. (1974)]. A good example of the insensitivity of the kinetics to strand mobility is the hybridization of DNA to complementary strands immobilized on nitrocellulose, which occurs as rapidly as DNA hybridization in solution (Gillespie & Spiegelman, 1965). It

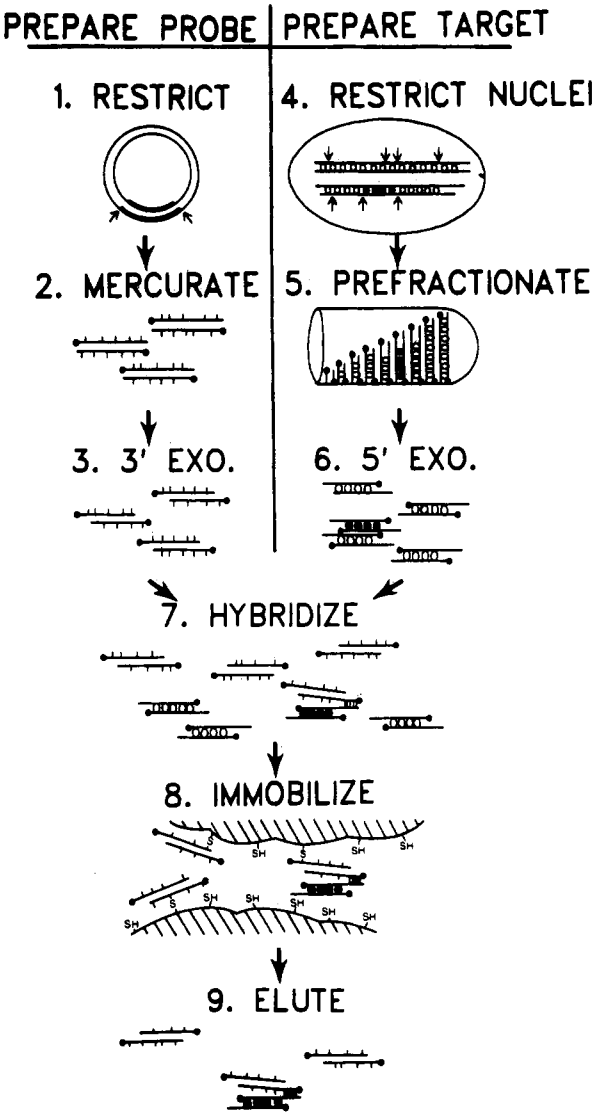

FIGURE 1: Schematic diagram of the nucleoprotein hybridization isolation procedure. (1) Restriction digestion of a recombinant plasmid. Arrows depict restriction sites, and the dark solid line represents an inserted eukaryotic sequence. (2) Mercuration of the probe DNA. Slashes represent sites of mercuration, and solid dots represent 5′-phosphates. (3) 3′-Exonuclease digestion of the mercurated probe leaving 5′ single-strand tails. (4) Restriction endonuclease digestion of nuclei to produce soluble chromatin. Nucleosomes are depicted as circles. The target sequence containing chromatin fragment is shown as closed circles. (5) Prefractionation by sedimentation velocity. (6) 5′-Exonuclease digestion of the chromatin fragments to produce 3′ single-strand tails. (7) Hybridization of the probe to the target chromatin fragment. Connecting lines represent base pairing between the 5′ tails of the probe and the 3′ tails of the target chromatin fragment. (8) Immobilization of the probe and probe–target hybrids on the surface of a sulfhydryl-Sepharose bead. (9) Elution of the probe and probe–target hybrids with reducing agents.

is reasonable to expect, therefore, that as long as chromosomal DNA has the same physical properties as naked DNA, the attachment of the chromosomal DNA to histones should not interfere significantly with DNA renaturation.

The existing data are consistent with the nucleosomal DNA being loosely bound to the surface of the octamer core and relatively free in the "linker" regions between nucleosome cores. Diffraction studies have located the DNA on the outer surface of the core (Pardon et al., 1975; Richmond et al., 1984). ^{31}P NMR studies by Shindo et al. (1980) revealed that nucleosomal DNA is highly mobile on the surface of nucleosomes. Thermodynamic data showed that the temperature, enthalpy, and cooperative length for melting of nucleosome core DNA are very similar to those properties of naked DNA (Weischet et al., 1978). In short, there are no results indicating that hybridization of DNA to chromatin should be less favored kinetically or thermodynamically than hybridization of DNA to DNA.

Several factors do complicate nucleoprotein hybridization, however. First, some of the chromosomal DNA must be made single stranded under mild conditions. We have solved this problem by digesting the chromatin with a strand-specific exonuclease. Second, the nucleoprotein hybridization reaction cannot be performed under the same conditions of high salt and temperature that are optimal for DNA renaturation. We have chosen conditions of 0.1 M salt and 37 °C to reduce the potential for protein migration or loss and have used a large concentration of mercurated probe in order to compensate for the reduction in reaction rate under these conditions. Third, during hybridization, nonspecific interactions might occur that could result in loss of proteins from the target chromatin, accretion of extraneous proteins onto the target chromatin, or contamination of the target chromatin with significant amounts of nonspecific chromatin fragments. The experiments to be described have established that these nonspecific reactions are insignificant.

Experimental Procedures

Reagents. Concentrations of enzymes are given in units (as measured by the suppliers) per microgram of DNA, standardized to the assay conditions of Bethesda Research Laboratories. Thiopropyl-Sepharose 6B was either purchased from Pharmacia or prepared from Sepharose 6B (Pharmacia) according to Axen et al. (1975).

The commonly used buffers are listed below. Additions or changes are described in the text. Homogenization buffer consisted of 15 mM NaCl, 60 mM KCl, 15 mM *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (Hepes)[1] (pH 7.3), 0.5 mM spermidine, 0.15 mM spermine, and 1 mM EDTA. Magnesium buffer was 60 mM KCl, 15 mM NaCl, 15 mM Pipes (pH 6.5), and 3 mM $MgCl_2$. Magnesium lysis buffer was magnesium buffer containing 0.1% Nonidet P-40. Chromatin digestion buffer was composed of 50 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.5 mM $MgCl_2$, and 1 mM 2-mercaptoethanol. λ exonuclease buffer consisted of 67 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, and 1 mM 2-mercaptoethanol. Hybridization buffer was 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.02% sodium azide. Tris–EDTA (TE) consisted of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

All reagents were screened in order to ensure that unwanted nucleases and proteases were not present, according to Workman & Langmore (1985). Except where noted, all the nuclear and chromatin preparations were carried out at 0–4 °C, except the chromatography and electrophoresis, which were carried out at room temperature.

Preparation of SV40 Minichromosomes. Minichromosomes were prepared by the method of M. Quasney and L. Lutter (unpublished results) which was a modification of the technique of Varshavsky et al. (1976). BSC-40 cells were grown to confluence in 9-cm plastic plates and then infected with wild-type SV40 virus (strain 776) to a multiplicity of infection of 5–10. For DNA labeling, 100 μCi of [^{3}H]thymidine or 30 μCi of [^{14}C]thymidine was added per plate at 24 h after infection. For protein labeling, cells were usually methionine-starved for 1.5 h at 48-h postinfection, after which 300 μCi of [^{35}S]methionine was added per plate.

At 54 h after infection, the cells on each plate were washed once in 5 mL of 140 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, and 25 mM Tris-HCl (pH 7.9), then scraped off the plate, and pelleted for 5 min at 1000*g*. The cells were lysed by resuspension followed by centrifugation (5 min at 1000*g*) in 5 mL of 15 mM NaCl, 60 mM KCl, 2 mM $MgCl_2$, 15 mM Tris-HCl (pH 7.9), 1 mM 2-mercaptoethanol, 0.1% Triton X-100, 340 mM sucrose, and 0.1 mM PMSF. Nuclei were lysed by resuspending three plates of nuclei in 300 μL of TE with 1 mM iodoacetate and 0.1 mM PMSF. In some preparations, iodoacetate was omitted, and aprotinin was added at a concentration of 0.17 unit/mL. After 1 h, the nuclei were pelleted for 10 min at 10000*g*. The supernatant was loaded onto a 13.5-mL, 15–30% (v/v) glycerol gradient in TE and centrifuged at 40 000 rpm for 200 min in a Beckman SW40 rotor. The gradient fractions containing the major rapidly sedimenting band of SV40 were pooled and dialyzed into λ exonuclease buffer or into chromatin digestion buffer for restriction endonuclease digestion. SV40 minichromsomes were usually digested with *Kpn*I (20 units/μg) or *Bam*HI (5 units/μg) for 3 h at 37 °C.

Preparation of Sea Urchin Embryo Chromatin. Gametes were collected from *Strongylocentrotus purpuratus* by coelomic injection of 1 mL of 0.5 M KCl. Ten milliliters of eggs was fertilized in 400 mL of synthetic seawater containing 1 mM 3-amino-1,2,4-triazole (Showman & Foerder, 1979). Embryos were washed twice with 400 mL of synthetic seawater suspended in 1 L of synthetic seawater containing 100 mg/L streptomycin sulfate and then incubated at 15 °C; 1 mCi of [^{3}H]thymidine or [^{3}H]leucine was added 5 min after the incubation was begun. When protein was to be labeled, 7 mCi of [^{35}S]methionine was added 6 h after the incubation was begun. After 18 h of development, the newly hatched blastulas were allowed to settle at 4 °C and resuspended in 50 mL of homogenization buffer containing 0.3 M sucrose, 1 mM iodoacetate, and 0.1 mM PMSF. Embryos were pelleted at 50*g*, resuspended in 24 mL of the same buffer, homogenized by 50 strokes with a Teflon pestle homogenizer, and then mixed with 2.1 volumes of homogenization buffer containing 2.3 M sucrose, 1 mM iodoacetate, and 0.1 mM PMSF. Twelve milliliters of the homogenates was layered over 1 mL of homogenization buffer containing 2.3 M sucrose and centrifuged in an SW40 rotor for 20 min at 40 000 rpm. The nuclear bands were drawn off the 2.3 M sucrose layers, pooled, diluted to 10 mL with magnesium lysis buffer containing 1 mM iodoacetate and 0.1 mM PMSF, and pelleted 10 min at 1000*g*.

[1] Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; kbp, kilobase pair(s); PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TBE, Tris–borate–EDTA; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid).

Nuclei were washed a second time in 10 mL of the same buffer and a third time in 10 mL of magnesium buffer. The nuclei were then resuspended to A_{260} = 2 in chromatin digestion buffer and digested with a restriction endonuclease at 5 units/μg of DNA for 3 h at 37 °C. Nuclear debris was removed by centrifugation for 30 min at 1000*g*. Depending on the restriction endonuclease used, 20–50% of the chromatin was recovered in the supernatant (Workman & Langmore, 1985). When necessary, the chromatin was concentrated by dialysis against buffer containing 60% sucrose (Workman & Langmore, 1985).

λ Exonuclease Digestion. Chromatin (20–50 μg/mL) was digested for 3 h at 37 °C with 4 units of lambda exonuclease per microgram of DNA in λ exonuclease buffer or chromatin digestion buffer. Digestions were stopped by adding 0.01 volume of 0.25 M EDTA (pH 8.0). The extent of digestion was assayed by acid solubility. Aliquots to be tested were diluted 100× with 0.2 mg/mL calf thymus DNA in TE and precipitated by adding $^1/_3$rd volume of 20% trichloroacetic acid. After 15 min at 0 °C, the suspension was centrifuged 15 min at 8000*g*.

Preparation of Mercury–DNA Probes. DNA was mercurated by using variations of the conditions employed by Dale & Ward (1975). The two plasmids used in this paper were pSVB3 (from W. Brockman), which contains the complete SV40 genome inserted into pBR322 at the *Bam*HI site, and pSp102 (from L. Kedes), which contains about 4 kbp of the *S. purpuratus* early histone gene repeat inserted at the *Eco*RI site of Col E1. Plasmid was restricted, extracted with chloroform/isoamyl alcohol (24:1), and dialyzed into 50 mM sodium acetate (pH 6.0). The DNA was diluted to a final concentration of about 100 μg/mL in 50 mM sodium acetate (pH 6.0) plus 20 mM mercury acetate (final pH 4.8).

Mercuration was carried out for 1 h at 50 °C in order to limit unwanted fragmentation of the probe. The reactions were stopped by the addition of 0.1 volume of 5 M NaCl and 0.1 volume of 0.25 M EDTA. Excess mercury was removed by dialysis against 20 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA for at least 4 h, followed by dialysis overnight against the same buffer containing 20 μM 2-mercaptoethanol. The mercurated DNA was then diluted with 0.1 volume of 0.5 M Tris-HCl (pH 8.0), 50 mM $MgCl_2$, and 0.2 M 2-mercaptoethanol, warmed to 37 °C, and digested with 250 units/mL exonuclease III for 30 s before addition of 0.1 volume of 0.25 M EDTA. The Hg–DNA was extracted once with chloroform/isoamyl alcohol, concentrated by dialysis against hybridization buffer with 60% sucrose, and then dialyzed against hybridization buffer.

Hybridization Reactions. All hybridization reactions were done at 37 °C in hybridization buffer, or in 100 mM NaCl, 10 mM EDTA (pH 8.0), and 0.02% sodium azide with identical results. The probe concentration was 100 μg/mL. Incubations were done in a 37 °C warm room by using tightly capped 1.5-mL polypropylene centrifuge tubes with their tips partly submerged in a beaker of water. Evaporation of water from the beaker kept the samples slightly cooler than 37 °C, thus preventing refluxing of the samples during hybridization. Aliquots were removed at the times indicated, diluted with running buffer to a final volume equal to that of the affinity columns, and immediately assayed on thiol-Sepharose columns.

Thiopropyl-Sepharose Chromatography. Thiopropyl-Sepharose columns (0.5–1 mL) were prewashed with 10 mL of hybridization buffer containing 1 mg/mL BSA and then with 10 mL of running buffer, consisting of hybridization buffer containing 0.05% Nonidet P-40, or 0.1 mg/mL BSA, to reduce nonspecific binding. Each aliquot of the hybridization solution was diluted with one column volume of running buffer and slowly loaded onto the column over a period of 1 h. The column was then washed with 10 mL of running buffer. The radioactive chromatin removed from the column during the loading and washing steps was denoted the "unbound" fraction. The mercurated probe and hybridized chromatin were then eluted with 10 mL of elution buffer (consisting of running buffer with 0.2 M 2-mercaptoethanol) and denoted the "eluted" fraction. Usually 5–30% of the bound hybrids could not be removed from the column due to irreversible binding of mercurated probe. Each fraction was counted in an aqueous counting scintillant. Each isotope activity was calculated by solving the obvious stimultaneous equations, including the background-corrected dual-channel counts and quench data for each isotope. The accuracy of this procedure was experimentally verified.

Since the mercurated probes were present in about 10-fold excess over the restricted minichromosomes, the hybridization reactions should follow pseudo-first-order kinetics. The fraction of restricted chromatin molecules unbound to the column (and therefore not hybridized to probe) should be given by the equation:

$$\frac{U(t) - U(\mathrm{f})}{U(0) - U(\mathrm{f})} = e^{-kPt} = e^{-0.69t/t_{1/2}} \qquad (1)$$

where $U(t)$ = the fraction of unbound restricted SV40 at time t, $U(0)$ = the initial fraction of unbound restricted SV40, $U(\mathrm{f})$ = the fraction of unbound restriction SV40 at infinite time, k = the reaction rate constant, P = the probe concentration, and $t_{1/2}$ = the pseudo-first-order half-life for the reaction under standard conditions ($P \approx 1.5 \times 10^{-8}$ M).

$U(t)$ and $U(0)$ were directly determined from the percentages of the SV40 radioactivity that were not bound to the columns, whereas $U(\mathrm{f})$ was determined by trial and error fitting of the data from each reaction to a straight line on a semilogarithmic plot. The asymptotic yield of each reaction was simply $1 - U(\mathrm{f})$. k and $t_{1/2}$ were determined from the slope of the straight line.

Electrophoresis and Fluorography. All restriction endonuclease digestions of plasmids and SV40 minichrosomes were checked by 0.7% agarose–TBE gels according to Maniatis et al. (1982). In SDS–PAGE analysis of column fractions, the chromatin was precipitated with 2 volumes of ethanol at −70 °C, pelleted, dried, and resuspended in SDS sample buffer (5% glycerol, 1% SDS, and 1% 2-mercaptoethanol). Greater than 90% of the protein was recovered by this procedure. The samples were run on 15% acrylamide/0.4% methylenebis-(acrylamide) gels (Laemmli, 1970), fixed, and stained for 1 h in 50% ethanol, 10% acetic acid, 5% formaldehyde, and 0.2% Coomassie blue, followed by overnight in 50% ethanol, 10% acetic acid, 0.5% formaldehyde, and 0.02% Coomassie blue. The gels were destained 1 h in 30% ethanol/10% acetic acid and 1 h in 10% ethanol/10% acetic acid. The gels were infiltrated wtih EnHance (New England Nuclear) according to instructions, dried, and fluorographed with screens at −70 °C against preflashed X-ray film (Laskey & Mills, 1975). Photographs of stained protein and DNA gels and fluorograms of gels were quantitated with a Joyce-Loebl Mark III microdensitometer.

Electron Microscopy. The chromatin samples used for microscopy (0.1–0.5 μg of chromatin in 0.1 mL of buffer) were first excluded from a 1-mL column of Sepharose 4B that had been preequilibrated with 5 mM Hepes (pH 7.0) and then fixed with 0.5% glutaraldehyde for 8–16 h at 0 °C. The

samples were prepared for microscopy by a modification of the technique of Thoma & Koller (1977). The microscopy was performed at a direct magnification of 30000–70000× in a JEOL JEM 100-B electron microscope operated at 100 kV.

RESULTS

Efficient Exposure of Specific Single-Stranded Sequences at the Termini of Chromatin Molecules. To prepare specific genes for nucleoprotein hybridization, the genes must first be cleaved at specific sequences, solubilized, and treated with a strand-specific exonuclease. This section will describe the processing of the SV40 minichromosomes that were used in subsequent nucleoprotein hybridizations. The conditions used are identical with those we have found optimal for the processing of chromatin from nuclei (Workman & Langmore, 1985).

There are two advantages to using restriction enzymes to cut and solubilize genes for isolation. First, the genes are released as discrete fragments which can be prefractionated on the basis of size (Workman & Langmore, 1985). Second, the specific genes will have specific terminal sequences that can be exposed by λ exonuclease digestion and hybridized to mercurated DNA of low complexity, as shown in the following sections. In order achieve the required efficiency and specificity of these digestions, we used a low concentration of magnesium (0.5 mM), which gave maximal accessibility and solubility of cellular chromatin without compromising the specificity of cleavage or structural integrity of the chromatin (Workman & Langmore, 1985).

For the SV40 experiments to be described, we chose to concentrate on two cleavage sites; (1) the *Kpn*I site, which is near the origin of replication and has been found to be exceptionally sensitive to nucleases; and (2) the *Bam*HI site, which is within the VP1 gene and has been found to have ordinary nuclease sensitivity (Varshavsky et al., 1979). Electrophoresis of the DNA after restriction of the minichromosomes showed that 50–70% of the *Kpn*I sites and 40–70% of the *Bam*HI sites were cut after 5-min digestions with 20 and 5 units/μg of enzyme, respectively. SDS–PAGE and electron microscopy were used to confirm that the cleaved SV40 molecules had not been damaged during the digestions (to be shown).

The next step in processing the minichromosomes was to expose a significant number of terminal nucleotides as single-stranded DNA. Prunell & Kornberg (1978), Riley & Weintraub (1978), and Herman et al. (1981) showed that exonuclease III was able to trim the 3′ strand of nucleosomal linker and core DNA. We chose to avoid use of exonuclease III due to the observation that it was capable of making double-strand cuts in nucleosomes (Riley & Weintraub, 1978).

λ exonuclease is known to digest the 5′ strand of double-stranded DNA in a highly processive manner (Little, 1967; Thomas & Olivera, 1978). Using an excess of λ exonuclease in order to reduce the effects of processivity and compensate for nonoptimal pH, we could digest restriction-digested chromatin to more than 10% acid solubility, if desired.

The kinetics of digestion of restricted minichromosomes are shown in Figure 2. Characteristically, there was a rapid initial digestion followed by a slow constant digestion. Interestingly, the rate of digestion at the *Kpn*I site, adjacent to the "open" region of the minichromosomes, was only slightly faster than that at the *Bam*HI site. Incubation of chromatin without added enzyme produced less than 0.3% acid solubility. Incubation of unrestricted minichromosomes with λ exonuclease for 3 h resulted in only 1.5% acid solubility, presumably due to digestion at internal sites in the 30% of the SV40 that had been isolated in the nicked form. Electrophoresis of the denatured SV40 DNA after λ exonuclease treatment showed negligible endonucleolytic degradation (not shown). λ exonuclease was shown to be equally effective in digesting the termini of restriction fragments of cellular chromatin, as seen in Figure 2.

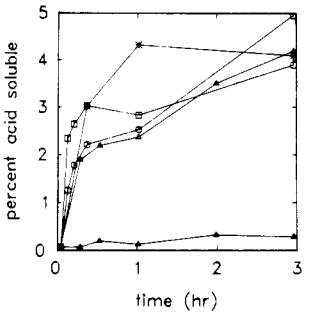

FIGURE 2: Release of acid-soluble thymidine from [^{14}C]- or [^{3}H]-thymidine-labeled chromatin fibers by λ exonuclease. Samples were digested and the acid solubilities tested at the indicated times as described under Experimental Procedures. Shown is the percent acid-soluble dpm as a function of time for *Kpn*I-digested SV40 minichromosomes (□), *Bam*HI-digested SV40 minichromosomes (○), *Hind*III-digested sea urchin embryo chromatin (asterisks), *Eco*RI-digested chicken erythrocyte chromatin (△), and a control incubation of *Eco*RI-digested chicken erythrocyte chromatin without added λ exonuclease (▲).

The observed 3–4% acid solubility of the minichromosomes is most easily interpreted as an average digestion of 300–400 nucleotides at each terminus (assuming 50% efficiency of restriction). Thus, it is likely that the exonuclease is capable of digesting beyond the first nucleosome at each terminus. This hypothesis is consistent with our measurements of the lengths and S1 endonuclease susceptibilities of the λ exonuclease treated chromatin (unpublished data). It is not inconceivable that nucleosomes were present on the putative single-stranded termini of the chromatin fragments, since there have been reports of stable nucleosomes bound to single-stranded DNA (Palter et al., 1979; Cafferelli et al., 1983).

Minichromosome Hybridization with Hg–pSVB3 and Assay by Thiopropyl-Sepharose Chromatography. Can the processed SV40 chromatin hybridize to complementary mercurated DNA? [^{3}H]Thymidine-labeled minichromosomes were cut with *Kpn*I (to 69% form III) and then digested with λ exonuclease (to 4% acid solubility). To distinguish between the effects of the SV40 chromosomal proteins upon λ exonuclease digestion and upon hybridization, the processed minichromosomes were separated into two aliquots. The first aliquot was dialyzed into hybridization buffer and then incubated as chromatin with 100 μg/mL Hg–pSVB3, according to the procedure described under Experimental Procedures. The second aliquot was stripped of protein by phenol extraction before dialysis and hybridization with Hg–pSVB3.

The amounts of SV40 hybridized to mercurated pSVB3 after various reaction times were determined from the amount of tritium that bound to thiopropyl-Sepharose, as shown in Figure 3. Surprisingly, the rate and extent of the DNA–DNA hybridization were identical with the rate and extent of nucleoprotein–DNA hybridization. Therefore, the presence of nucleosomes on the SV40 did not seem to interfere with hybridization.

Since the mercurated pSVB3 probe was about 10× more concentrated than the SV40 in these experiments, the hybridization reactions should have exhibited pseudo-first-order kinetics. Figure 4 is a semilogarithmic presentation of the hybridization data previously shown in Figure 3. Clearly, the

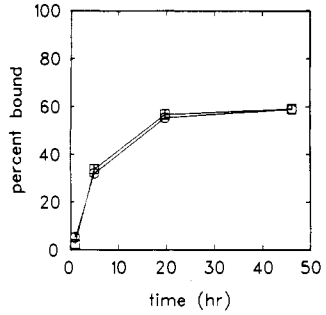

FIGURE 3: Hybridization of SV40 to mercurated pSVB3, as assayed by thiopropyl-Sepharose binding. The percentage of the restricted SV40 is plotted vs. the time of incubation with 100 μg/mL mercurated, exo III treated probe. [^{3}H]Thymidine-labeled minichromosomes were *Kpn*I and λ exonuclease treated and then subsequently directly hybridized to mercurated probe (○) or extracted with phenol and chloroform first and then hybridized to mercurated probe (□).

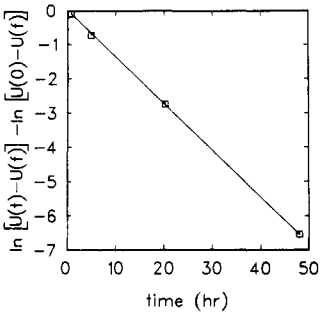

FIGURE 4: Semilogarithmic plot of the SV40 minichromosome hybridization data shown in Figure 3. Plotted is the decay of unbound *Kpn*I-digested SV40 minichromosomes vs. time of hybridization. The straight line follows the pseudo-first-order kinetics expected under our conditions of excess probe.

first-order rate equation provides an excellent description of the reaction kinetics. The fact that almost every chromatin hybridization reaction studied showed pseudo-first-order kinetics from the start to the apparent end of the reaction is strong evidence that all the reactive chromatin molecules hybridized at the same rate. Thus, either the processed chromatin molecules were very homogeneous in structure or any heterogeneity that might have existed in the length or structure of the single-strand termni did not affect the rate of reaction.

The $t_{1/2}$ values of the reactions shown in Figures 3 and 4 were 4.5 h for the DNA hybridization and 4.1 h for the chromatin hybridization. Since the probe concentration was $\approx 1.5 \times 10^{-8}$ M, the rate constant, k, was $\approx 1 \times 10^7$ L mol^{-1} h^{-1}. The asymptotic yields were 76%, meaning that only about 24% of the SV40 would not have bound to the columns even at the completion of the first-order reaction. The amount of unbound chromatin is comparable to the 20% of mercurated probe that did not bind to the thiopropyl-Sepharose columns in separate experiments (results not shown). Thus, it is possible that better than 95% of the chromatin had hybridized in solution.

Why were the kinetics of the minichromosome and DNA hybridization reactions in Figure 3 so similar? Were the minochromosome proteins lost or rearranged during the enzymatic and hybridization reactions? We can exclude this possibility with the following results: (a) the slow rate of λ exonuclease digestion of restricted minichromosomes indicated the persistence of an impediment (presumably proteinaceous) to enzymatic digestion of the SV40 termini; (b) electron microscopy demonstrated the presence of a normal complement of nucleosomes on the λ exonuclease treated and hybridized minichromosomes (data to be shown); and (c) SDS–PAGE demonstrated that the SV40 histone and non-histone proteins remained on the SV40 molecules during the processing and hybridization steps (data to be shown). Therefore, we conclude that nucleoproteins were capable of renaturation at a rate comparable to that of identically processed, naked DNA.

Table I: Nonspecific Reactions of SV40 Minichromosomes with Mercurated Probe

type of probe	conditions: exo III treatment to probe	conditions: λ exonuclease treatment of SV40	incubation time (h)	% SV40 bound to SH-Sepharose
pSVB3	+	+	0	1.9
pSVB3	+	+	24	56.0
pSp102	+	+	24	2.0
pSp102	–	+	24	2.4
pSVB3	+	–	12	1.5
pSVB3	–	+	24	4.0

Specificity of the Binding of Minichromosomes to pSVB3. It is reasonable to interpret the results above as evidence that the minichromosomes hybridized to the pSVB3. To prove that this interpretation is correct, we needed to demonstrate that the reaction (a) was sequence specific, (b) depended upon the presence of single-stranded termini on the SV40, and (c) depended upon exo III digestion of the probe.

Table I shows the results of experiments that demonstrate the specificity of the reaction between the single-stranded tails of the probe and target molecules. All processing, incubations, and chromatography were under standard conditions.

Characteristic of the sequence-specific reaction was the binding of a significant fraction of the input SV40 to the columns. In the case shown in Table I, about 56% of the input SV40 was bound after a 24-h incubation, representing in this case about 95% of the linear minichromosomes. When the specific probe was mixed with the processed SV40 but not incubated for a significant time, only about 2% of the SV40 became bound to the column. When the nonhomologous probe pSp102 was used, only about 2% binding of SV40 was observed. Elimination of either exo III treatment of the Hg–pSVB3 or λ exonuclease treatment of the minichromosomes also eliminated significant binding of the SV40. Thus, our observations about the reaction between the the Hg–pSVB3 and the minichromosomes were consistent with the anticipated mechanism, that of a sequence-specific hybridization between the single-stranded termini of the probe DNA and target chromatin molecules.

Hybridization of Minichromosomes with Termini at the BamHI Site. It is possible that the *Kpn*I site of SV40 is exceptionally reactive, due to the special character of the chromatin near the origin of replication of the minichromosomes. This region of the genome has been found to be devoid of nucleosomes in about 25% of the molecules examined in the electron microscope (Saragosti et al., 1980) and to be unusually accessible to endogenous and exogenous nonspecific endonucleases (Scott & Wigmore, 1978; Waldeck et al., 1978; Nedospasov & Georgiev, 1980). Therefore, it was important for us to be reassured that other sites on the SV40 minichromosome could also be hybridized to mercurated probe molecules. We chose the *Bam*HI site since it is located in the structural gene for VP1.

We processed and hybridized in parallel two aliquots of [^{3}H]thymidine-labeled SV40. The first aliquot was digested for 3 h with 20 units/μg of *Kpn*I (to about 70% form III), digested for 3 h with 4 units/μg of λ exonuclease, concentrated against sucrose (to 18 μg/mL), and incubated with *Bam*HI,

Table II: Results of Mixed SV40–Sea Urchin Hybridization

before fractionation		after fractionation					
*Kpn*I-cut SV40[a] (ng)	*Kpn*I-cut sea urchin chromatin (ng)	*Kpn*I-cut SV40[b] (ng)	*Kpn*I-cut sea urchin chromatin[b] (ng)	enrichment of SV40[b,c]	purification of SV40[b,d]	asymptotic yield[e] (%)	$t_{1/2}$[e] (h)
12		7.7				79	3.1 ± 1
19	285	12	1.7	106	14.0	65	4.5 ± 1

[a] Determined from the total amount of SV40, and the agarose gel electrophoresis result that 66% of the SV40 DNA was form III (linear). [b] After 24-h hybridization. [c] The *x*-fold increase in the ratio of *Kpn*I-cut SV40 chromatin to non-SV40 chromatin. [d] The *x*-fold increase in the ratio of *Kpn*I-cut SV40 chromatin to total chromatin. [e] As determined previously, assuming first-order kinetics.

*Kpn*I-cut mercurated pSVB3 (at 100 μg/mL). The second aliquot was digested for 3 h with 20 units/μg of *Bam*HI (to about 70% form III), digested for 3 h with 4 units/μg of λ exonuclease, concentrated against sucrose (to 18 μg/mL), and incubated with the same mercurated pSVB3 (at 100 μg/mL). Aliquots were taken from both reactions at 0, 2, 4, 8, 12, 24, and 48 h of incubation and subjected to chromatography. Hybridization at the *Kpn*I site occurred with a $t_{1/2}$ of 2.0 h and an asymptotic yield of 75%. Hybridization at the *Bam*HI site occurred with a $t_{1/2}$ of 1.6 h and an asymptotic yield of 75%. Thus, the rate and extent of hybridization were essentially identical for molecules terminated at the *Kpn*I site, which is in the open region near the origin of replication, and at the *Bam*HI site, which is in a structural gene.

Recovery of SV40 Minichromosomes from a Mixture with Sea Urchin Embryo Chromatin. In order to test the specificity and yield of the nucleoprotein hybridizations under the conditions necessary for isolation of a specific cellular gene, we intentionally mixed SV40 with an excess of sea urchin chromatin and then tried to reisolate the minichromosomes by nucleoprotein hybridization.

[^{14}C]Thymidine-labeled SV40 was digested with *Kpn*I. One aliquot was mixed with a 15-fold excess of [^{3}H]thymidine-labeled sea urchin chromatin that had been solubilized with *Kpn*I. A second aliquot remained unmixed. Both aliquots were digested with λ exonuclease and then hybridized to *Kpn*I-cut mercurated pSVB3. The results of the thiopropyl-Sepharose chromatography are shown in Figure 5. The minichromosomes hybridized well in both samples. The presence of sea urchin chromatin decreased the amount of SV40 that could not be eluted, presumably due to saturation of the nonspecific binding sites on the columns. Analysis of the kinetics and yield (Table II) shows the neither the rate nor the extent of hybridization was significantly affected by the presence of the excess sea urchin chromatin. After 48 h of mixed hybridization, the [^{14}C]thymidine-labeled SV40 in the eluted fraction was 88% pure with a yield of 63%. This represents a 14-fold purification of the SV40, and a 115-fold enrichment of SV40 relative to the nonspecific, sea urchin chromatin.

We strongly believe that the enrichment of SV40 in the mixed-hybridization experiments was due to sequence-specific hybridization of the mercurated pSVB3 to the SV40 minichromosomes and not due to a physical artifact. The sequence dependence of the SV40 reaction was shown above. The enrichment was not due to a size difference between the SV40 and the sea urchin chromatin, since both were of comparable size (data not shown). In addition, the nonspecific binding of sea urchin chromatin to the columns was found to be independent of molecular weight from less than 5 kbp to over 20 kbp (data not shown). In independent experiments, we enriched minichromosomes from mixtures with restricted chicken erythrocyte chromatin, with results very similar to those achieved with the sea urchin chromatin (data not shown).

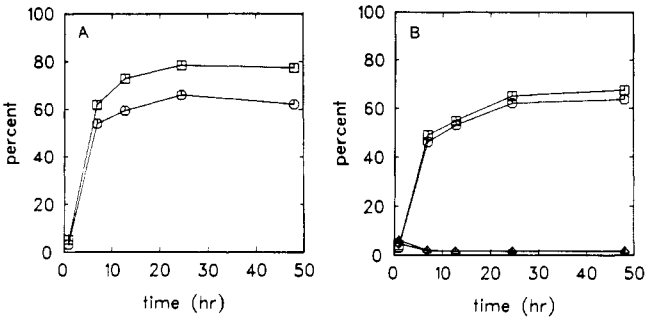

FIGURE 5: Hybridization of minichromosomes alone (A) and in a mixture with sea urchin chromatin (B). [^{14}C]Thymidine-labeled SV40 minichromosomes were digested with *Kpn*I. 0.7 μg of restricted SV40 was prepared for a mixed-hybridization reaction by mixing it with 10 μg of [^{3}H]thymidine-labeled sea urchin chromatin that had previously been solubilized from nuclei by digestion with 5 units/μg of *Kpn*I. The second 0.7-μg aliquot of SV40 was kept for a control hybridization reaction. The mixed and unmixed aliquots of SV40 were each digested with 4 units of λ exonuclease per microgram of total DNA. 1.4% of the [^{14}C]thymidine and 3.0% of the [^{3}H]thymidine were released as acid-soluble counts from the mixed sample. 4.0% of the [^{14}C]thymidine was released from the unmixed sample. Both samples were dialyzed and concentrated into hybridization buffer containing 50 μM 2-mercaptoethanol. Hg–PSVB3 probe in hybridization buffer was added to the samples and incubated begun under the following conditions: for the mixed sample, 0.19 μg of linear SV40, 2.85 μg of sea urchin chromatin, and 10 μg of mercurated probe in 100 μL; for the unmixed sample, 0.120 μg of linear SV40 and 2.66 μg of mercurated probe in 26.6 μL. At various times of hybridization, 5- and 10-μL aliquots of the unmixed and mixed samples, respectively, were diluted with 500 μL of running buffer containing 100 μg/mL BSA and subjected to thiopropyl-Sepharose chromatography. Aliquots taken at the times shown were assayed on thiopropyl-Sepharose to determine the percent SV40 bound (□) and eluted (○). In the mixed hybridization, aliquots were also tested for percent sea urchin chromatin bound (△) and eluted (◇).

Measurements of the Loss of SV40 Chromosomal Proteins during the Isolation of Minichromosomes. To effectively use nucleoprotein hybridization to identify the proteins associated with specific genes in cells, the isolation methods must not disrupt chromosomal proteins by causing (a) proteolytic degradation, (b) loss of proteins from the gene of interest, (c) accretion of extraneous proteins onto the gene of interest, or (d) extensive rearrangement of proteins. We have experimentally determined the magnitudes of the first three of these effects using the SV40 test system. Protein rearrangement within a gene is a less important effect, which will be given attention under Discussion.

In order to determine whether minichromosomal proteins were lost or degraded during any of the steps of our isolation procedure, we followed the fate of radioactively labeled SV40 proteins during mixed hybridizations in a background of excess sea urchin chromatin. [^{3}H]Thymidine- and [^{35}S]-methionine-labeled minichromosomes were processed with *Kpn*I and λ exonuclease and then added to a 16-fold excess of *Kpn*I-restricted sea urchin embryo chromatin. The mixture

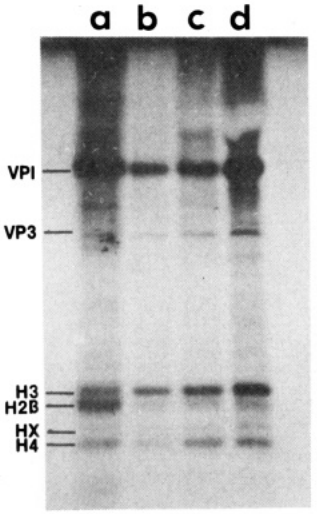

FIGURE 6: Distribution of proteins after the mixed hybridization of [^{35}S]methionine- and [^{3}H]thymidine-labeled SV40 with Hg–pSVB3 in the presence of a 15-fold excess of unlabeled sea urchin chromatin. The fluorogram is one of three used to evaluate the covalent integrity and the amounts of SV40 protein that had bound to the mercurated probe and sea urchin chromatin. The amount of sample loaded into each lane is given in nanograms of DNA. Lane a was loaded with a sample of a nuclear pellet from SV40-infected BSC-40 cells; lane b was loaded with the acid-soluble proteins released from 108 ng of glycerol gradient purified SV40; lane c was loaded with 61 ng of minichromosomes that were not bound to thiopropyl-Sepharose; and lane d was loaded with 94 ng of minichromosomes that were isolated by elution from the same column. The fluorograms are quantitatively interpreted in Table III. Lanes c and d show quenching of fluorescence at about 70K, due to the excess unlabeled BSA present.

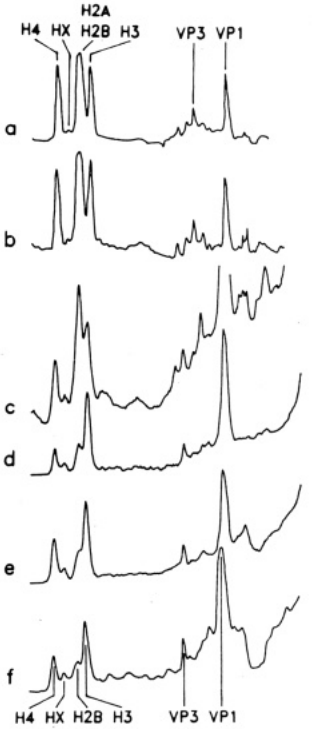

FIGURE 7: Densitometric tracings of the fluorogram shown in Figure 6, as well as the a Coomassie blue stained SDS–polyacrylamide gel. The Coomassie blue stained samples are nuclear pellet from infected cells (trace a) and glycerol gradient isolated SV40 (trace b). The fluorographed samples are nuclear pellet from infected cells (trace c), acid extract from glycerol gradient isolated SV40 (trace d), unbound fraction from the SH-Sepharose column (trace e), and eluted fraction from the SH-Sepharose column (trace f). The traces of the fluorogram have been normalized to a constant amount of ^{3}H-labeled DNA. The Coomassie-stained histones have the expected relative intensities. The unbound and the eluted protein patterns closely resemble those of the infected nuclei and freshly isolated SV40. Thus, no substantial proteolysis or exchange has taken place (see text and Table III).

was concentrated and incubated under standard hybridization conditions with Hg–pSVB3. In addition, an unmixed hybridization reaction was performed as a control. The experimental protocol for both experiments was chosen to be the same as that used for the successful mixed hybridizations described in the previous section. After 6 h of hybridization at 37 °C, the reaction mixtures were fractionated by thiopropyl-Sepharose chromatography. At that time, 38% of the SV40 bound to the column. We used SDS–PAGE in order to compare the protein compositions of the chromatin before and after SV40 isolation.

Figure 6 is a fluorogram of the [^{35}S]methionine-labeled SV40 at different stages of the mixed-hybridization experiment. Figure 7 shows densitometer traces of several lanes of the fluorogram as well as the Coomassie blue stained gel containing SV40 minichromosomes and nuclear pellets from the same preparation. The prominent low molecular weight Coomassie blue stained bands were histones H4, H2A and H2B, and H3. There was also a small amount of a weakly stained, acid-soluble protein (perhaps a histone variant) that ran slight slower than H4, which we denote HX. A comparison of the stained gel and the fluorogram shows that histone H2A was not labeled with ^{35}S, in agreement with the results of Mann & Hunter (1979). The relative amounts of stain bound to the H4, H2A + H2B, and H3 bands were 1.0, 1.96, and 1.2, respectively. Thus, the relative amounts of the histones in the isolated SV40 were close to those expected. The nuclear pellet contained a small amount of a highly labeled protein that was not acid soluble and was not bound to the isolated minichromosomes. The two acid-soluble proteins at about 43 and 30 kilodaltons were identified as viral proteins VP1 and VP3, based on the results of Tegtmeyer (1974). These proteins are known to be bound to wild-type SV40 minichromosomes and to be labeled to high specific activity with [^{35}S]methionine. Although the histones comprise 80% of the Coomassie blue stained material, they contain only 20–30% of the radioactive label. Other non-histone proteins, such as T-antigen, were not resolved, due to the high percentage of acrylamide in these gels.

A qualitative comparison of the lanes shown in Figures 6 and 7 shows that the ^{35}S-labeled SV40 histones remained on the SV40 isolated by nucleoprotein hybridization and thiopropyl-Sepharose chromatography. The apparent relative amounts of H3, H2B, and H4 were the same in the acid-extracted SV40, in the unbound SV40, and in the eluted SV40. Table III shows the quantitative analysis of the SDS–PAGE fluorograms. The amounts of specific proteins per unit of DNA were measured in arbitrary units, by dividing the areas under the densitometer traces by the amount of tritium loaded onto each lane of the gel. Difficulties were obvious in the quantitation of the histone to DNA ratios for several samples. The nuclear pellets were depleted of DNA due to the insolubility of the genomic DNA in the SDS sample buffer. In addition, the glycerol gradient purified SV40 seemed to have an abnormally low histone to DNA ratio, perhaps due to the presence of a large quantity of unincorporated [^{3}H]thymidine, which could have been lost during the multiple steps of dialysis necessary for the processing of the chromatin. For the remainder of the samples, the histone to DNA ratio remained remarkably constant during the hybridization and chromatographic separations. Only the amount of VP1 varied substantially during the isolation steps. Since the relative amounts of H3, H2B, H4, and VP3 remained constant during isolation of the SV40, we can say with confidence that the eluted

Table III: SDS–PAGE Autoradiography of [^{35}S]Methionine- and [^{3}H]Thymidine-Labeled SV40–Sea Urchin Chromatin Mixed-Hybridization Samples

protein sample	histone:^{3}H[a] ratio	individual histone:total histone[a] ratio				VP3:histone[a] ratio	VP1: histone[a] ratio
		H4	HX	H2B	H3		
nuclear pellet	1.54[b]	0.23	0.032	0.49	0.26	0.065	1.7
gradient isolated	0.042[c]	0.15	0.040	0.21	0.60	0.048	5.2
acid extracted	0.047[d]	0.17	0.033	0.18	0.61	0.047	1.0
after processing	0.164	0.23	0.034	0.15	0.59	0.059	1.4
after hybridization	0.134	0.23	0.052	0.13	0.57	0.060	1.2
unbound on SH-Sepharose column	0.130	0.25	0.040	0.17	0.55	0.076	0.95
eluted from SH-Sepharose column	0.143	0.16	0.038	0.13	0.66	0.070	1.5

[a] The amount of protein is measured in arbitrary units of area (millimeters squared) under the densitometer tracing (1–2 specular density range) of the autoradiogram. The histone to DNA ratio is given as the ratio of the area divided by the dpm of ^{3}H loaded onto the gel. [b] A nuclear pellet of infected BSC-40 cells was taken after extraction of SV40 and suspended in 1% SDS. The majority of the high molecular weight DNA was not soluble in SDS, making this table entry incorrectly large. [c] This figure appears to be incorrect, perhaps due to contamination of the sample with free [^{3}H]thymidine. [d] This figure is low, probably due to incomplete extraction of the histones.

fraction contained >90% of the original amount of histone protein, as well as a comparable fraction of the non-histone VP3. However, VP1 seems to be labile, possibly due to binding to the mercurated pSVB3 or to the thiopropyl-Sepharose column.

The apparent lack of histone loss was also confirmed by a second protein transfer experiment with similar design, using a different preparation of minichromosomes, sea urchin chromatin, and probe. In these second experiments, the SV40 and sea urchin chromatin were mixed before the *Kpn*I and λ exonuclease treatments. Thus, the restriction and exonuclease reactions did not cause loss of histones from SV40.

The preservation of the native ratio of histones to DNA during the mixed-hybridization experiment is a critical indication that the nucleoprotein hybridization technique has the potential of isolating gene-specific proteins. Obviously, proteolysis has been ruled out, since the relative and absolute amounts of all three labeled histones have not changed during isolation. In addition, the possibility of protein exchange between the minichromosomes and either the sea urchin chromatin, the mercurated probe, or the solution has been almost completely eliminated. If SV40 histones had exchanged with the 16-fold excess of sea urchin chromatin, then the [^{35}S]methionine-labeled histones would have been concentrated in the unbound fraction. For instance, if histone exchange between SV40 and sea urchin chromatin had been complete, the ratio of histone to DNA in the eluted fraction should have been $^{1}/_{16}$ of the ratio in the unbound fraction. Our results exclude the occurrence of greater than 10% loss of SV40 histones to the sea urchin chromatin. Our results also exclude the possibility of extensive binding of SV40 histones to the mercurated probe. Since the probe was in 120-fold excess over all the SV40 chromatin, even a small tendency of the histones to bind to the probe would have been detected as a decrease in the histones in the unbound fraction. Loss of histones to the probe would have also resulted in changes in the sedimentation velocity of the minochromosomes, which were not observed in the unbound or the eluted samples (results not shown). Finally, loss of histones to the solution would have been detected as an increase in the histone content of the unbound fraction. Thus, we have excluded major loss or transfer of the SV40 proteins during nucleoprotein hybridization.

Transfer of Non-SV40 Proteins onto the Minichromosomes and Mercurated Probe during the Mixed-Hybridization Experiments. In order to confirm the lack of substantial transfer of sea urchin histones onto the mercurated probe and SV40, we performed an additional mixed-hybridization experiment. [^{3}H]Thymidine-labeled *Kpn*I-cut SV40 was mixed with an excess of [^{35}S]methionine-labeled *Kpn*I-cut sea urchin chromatin. According to standard protocol, the mixture was digested with λ exonuclease, incubated with mercurated pSVB3 probe, and separated on thiopropyl-Sepharose. The initial mixture contained 0.28 μg of restricted SV40 (as [^{3}H]thymidine) and 4.2 μg of sea urchin chromatin (as [^{35}S]-methionine). After hybridization, the first eluted fraction from the column contained 0.095 μg of SV40 contaminated with 0.046 μg of sea urchin protein. Thus, only 1.1% of the sea urchin proteins appeared in the eluted fraction. We believe that these proteins were bound to the probe or to the column, since a parallel incubation of pure sea urchin chromatin with mercurated probe also gave about 1% nonspecific binding (data not shown).

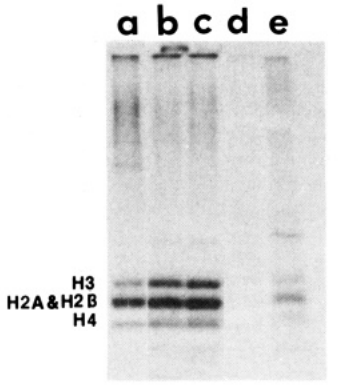

FIGURE 8: Fluorogram from SDS–PAGE of thiopropyl-Sepharose fractions from the mixed hybridization of [^{3}H]thymidine-labeled SV40 minichromosomes with a 15-fold excess of [^{35}S]methionine-labeled sea urchin chromatin. 4.2 μg of *Kpn*I-cut sea urchin chromatin (132 000 dpm/μg) was mixed with 0.28 μg of *Kpn*I-cut minichromosomes (412 000 dpm/μg). The mixtures were digested with λ exonuclease (to about 4% acid solubility), concentrated to 27 μg/mL, and incubated with 100 μg/mL processed pSVB3 for 14 h at 37 °C, according to standard protocol. Chromatography on thiopropyl-Sepharose was carried out as described previously. The samples are as follows: lane a, sea urchin nuclei; lane b, 4 ng of SV40 chromatin in the mixture with sea urchin chromatin before hybridization; lane c, 4 ng of SV40 chromatin in the mixture with sea urchin chromatin from the fraction unbound to thiopropyl-Sepharose; lane d, 7 ng of SV40 chromatin in the mixture with sea urchin chromatin from the fraction eluted from thiopropyl-Sepharose; lane e, 95 ng of SV40 chromatin in the mixture with sea urchin chromatin from the fraction eluted from thiopropyl-Sepharose. Since the sea urchin chromatin did not hybridize to the pSVB3, the eluted sample was highly depleted in sea urchin proteins. When greatly overloaded, the eluted sample shows native proportions of the labeled histones. In the eluted fraction, one non-histone protein is enriched relative to the histones.

Qualitative analysis of the SDS–PAGE results (Figure 8) shows that the labeled sea urchin histones were represented

normally in the unbound fractions from the SH-Sepharose but were not detectable in the lane loaded with a 2-fold excess of the eluted fraction. However, the lane containing a 24-fold excess of eluted SV40 showed that the relative amounts of each of the core histones in the eluted sample were about the same as those in the untreated sea urchin samples. To be more quantitative, the whole sea urchin nuclei were measured to have relative radioactivities of 0.14, 0.64, and 0.23 for H4, H2B, and H3, respectively. The unbound chromatography fractions were measured to have values of 0.14, 0.57, and 0.28 for the same core histones. In the eluted material, the ratios were 0.17, 0.62, and 0.23, respectively. Since the relative amounts of the core histones in the eluted fractions were comparable to those in the intact nuclei, the histone background was probably due to the sticking of intact sea urchin chromatin onto the column and probe, rather than selective transfer of certain proteins. Of course, we can say nothing about the exchange of H1, since it was not labeled. H1, however, is expected to exchange, based upon earlier reports (Thomas & Rees, 1983). The non-histone protein that ran behind H3, perhaps a high mobility group protein, was enriched about 3-fold in the eluted fraction, perhaps due to binding to the mercurated probe.

Electron Microscopy of the SV40 Minichromosomes during Isolation by Nucleoprotein Hybridization. We have done preliminary microscopic studies of SV40 minichromosomes in order to demonstrate the feasibility of performing structural studies on chromatin isolated by nucleoprotein hybridization as well as to confirm that the isolated material was structurally intact.

Figure 9 shows electron micrograph images of conventionally prepared SV40 minichromosomes prepared at different stages of isolation. The intact, gradient-isolated SV40 (panels A and B) shows the characteristic pattern of circular beaded molecules. After restriction of the minichromosomes with *Kpn*I (panels C and D), a large fraction of the molecules appear extended, presumably linearized by double-strand cleavage. After incubation with the mercurated pSVB3, and thiopropyl-Sepharose chromatography, the eluted fraction (panels E–F) contained molecules that were regularly studded with nucleosomes and have the expected length of SV40. In the background, there are many aggregated filamentous molecules that are presumably mercurated probe. These aggregated molecules show little evidence of a beaded, nucleosome-like structure. Separate examinations of mercurated probe showed similar heterogeneous structures. We conclude that the SV40 histones remained bound as nucleosomes to the isolated minichromosomes.

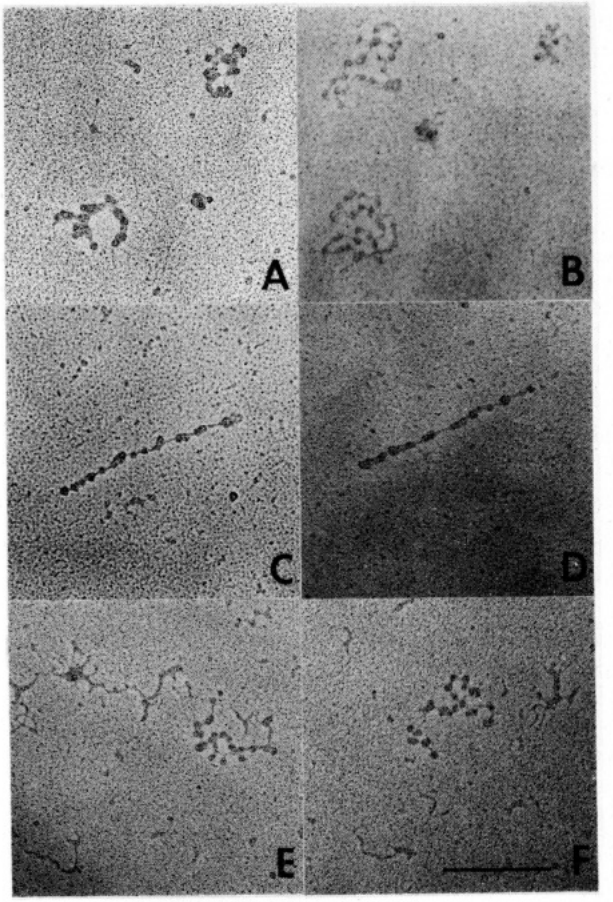

FIGURE 9: Electron micrographs of benzyldimethylalkylammonium chloride spread SV40 minichromosomes at different stages during isolation. Micrographs are of closed circular SV40 after isolation from BSC-40 cells (A and B), after digestion with *Kpn*I (C and D), and after reisolation by nucleoprotein hybridization and affinity chromatography (E and F). Magnification bar represents 200 nm.

Discussion

Status of the Nucleoprotein Hybridization Technique. The nucleoprotein hybridization technique has been developed to the extent that it is certain that a specific chromatin fragment can be efficiently restricted, λ exonuclease processed to expose 3′ single-stranded termini, stringently hybridized to complementary single strands on a mercurated DNA probe, and subsequently bound and eluted from thiopropyl-Sepharose. The yield of the hybridization step was usually 60–90% of the restricted SV40, and the overall yield of SV40 from the beginning to the end of the experiment was usually 10–15%. The typical half-time for the pseudo-first-order hybridization reaction was about 5 h at 37 °C in 0.11 M monovalent salt, with about 1.5×10^{-8} M probe molecules. In the mixed-hybridization experiments, we purified minichromosomes from a starting purity of 6% to a final purity of almost 90%. This purification seems to be primarily limited by the 0.2–2% nonspecific binding of the background sea urchin or chick chromatin to the thiopropyl-Sepharose and/or the mercurated probe. Fortunately, the rate and yield of the hybridization reaction do not seem to depend upon the particular restriction site chosen or upon the amount of non-SV40 chromatin present.

Our results constitute the first evidence that chromatin molecules can hybridize with DNA and signify the first isolation of specific genes as chromatin by a sequence-specific method.

In order to extrapolate our results to sequence-specific isolation of chromatin under other circumstances, we need to employ the concept of enrichment rather than purification. The degree of purification is the ratio of the final to the initial purity of the sample, and thus depends upon the fraction of SV40 in the initial mixture. In the case of our mixed-hybridization reactions (which began with about 6% SV40), we achieved purifications of about 14-fold. However, the chromatographic yield of SV40 was a constant fraction (about 70%) of the input SV40, independent of the amount of background chromatin. Likewise, the yield of nonspecific background was a constant fraction (about 0.6%) of the input nonspecific chromatin. The ratio of these percentages is equal to the sequence-specific enrichment, experimentally found to be about 115-fold after 48 h of hybridization. Unlike the value for purification, the value for enrichment can be used to predict the outcome of experiments with other initial conditions. Using the value of 115-fold enrichment, for example, an experiment begun with 0.5% SV40 and 99.5% nonspecific background is

predicted to yield 0.35% SV40 and 6% background, resulting in a final SV40 purity of almost 40% (an 80-fold purification).

Our use of SV40 minichromosomes as a test system gave us the unique opportunity to assay the fate of the SV40 proteins during the isolation. We are confident that less than 10% SV40 histone loss or exchange occurred during isolation. We can also say that there was only about 1% transfer of nonspecific (i.e., sea urchin) chromosomal proteins onto the mercurated probe and columns during the isolation. In addition, physical properties of the SV40 such as the sedimentation velocity and the appearance of nucleosomes in the electron microscope were unchanged by isolation.

The failure to observe serious protein exchange with the background chromatin or the mercurated probe under our mild incubation conditions was consistent with the reports of others. For instance, Germond et al. (1976) reported negligible loss of nucleosomes from SV40 minichromosomes during 30-min incubations at 37 °C with equal amounts of 100 μg/mL SV40 form I DNA, as long as the salt concentration was <700 mM NaCl. Cremisi et al. (1976) were unable to detect protein exchange after 2-h incubations of minichromosomes with DNA. Ilyin et al. (1971) studied histone and non-histone transfer from sheared chromatin to exogenous tRNA, DNA, and other chromatin. Little exchange of core histones and non-histones was found after 24-h incubations at 0.12 M NaCl.

We have not attempted to assay for lateral migration (sliding) of nucleosomes on SV40 during isolation. Future applications of the nucleoprotein hybridization technique are not anticipated to have difficulties with nucleosome migration, however. In particular, Cremisi et al. (1976) and Glotov et al. (1982) were unable to detect sliding of SV40 nucleosomes at 100–150 mM NaCl. When H1 was removed, sliding was noticed at 37 °C at >150 mM NaCl (Beard, 1978; Glotov et al., 1982).

Improvements of the Nucleoprotein Hybridization Technique. Before discussing the goal of isolating specific genes, it is appropriate to raise two issues of direct relevance to the achievement of that goal—the limitations of the existing technique and the improvements that can be made in order to make cellular gene isolation more feasible.

The major limitation of the isolation technique is the nonspecific binding of chromatin to the thiopropyl-Sepharose columns. Under favorable conditions, the background binding and elution of sea urchin chromatin from the columns are less than 0.1%. Under unfavorable conditions, the background chromatin in the eluted fraction can approach 3%. Thus, used in combination with gradient prefractionation, nucleoprotein hybridization is only capable of isolating highly reiterated genes. Different affinity techniques, employing probes containing biotinylated bases (Langer et al., 1981), polynucleotide terminal sequences, etc., seem necessary to increase the specificity of the isolations.

The transfer of chromosomal proteins and the nonspecific background could be reduced by reducing the molecular weight of the probe. The probes used in this paper have consisted of 10 kbp plasmids, with only about 400 bases of productive single-stranded DNA. Reducing the probe to just the 400 bases spanning the restriction site would decrease the total amount of DNA in the incubation mixtures by at least a factor of 50, thereby decreasing the number of nonspecific binding sites for chromosomal proteins on the probe by a similar factor. Reduction of the probe molecular weight should also increase the activity coefficient of the probe due to a reduction of steric hindrance (Wetmur & Davidson, 1968). These improvements could be achieved by use of in vitro transcription to produce mercurated or biotinylated probes (Melton et al., 1984).

Another potential difficulty in the present methodology is the large excess of probe DNA present in the eluted fraction, which could interfere with the detection or analysis of the isolated genes are chromatin. Interference during detection of the isolated gene fragments can be avoided by simply employing nonoverlapping sequences for the probe used for isolation and the probe used for detection. Interference of the mercurated probe with analysis of the protein or DNA of the isolated chromatin can be avoided by separating the mercurated probe from the chromatin molecules. This can be done by the use of mild single-strand nuclease digestion, followed by gradient centrifugation or a second cycle of thiopropyl-Sepharose chromatography. We have chosen, instead, to use RNA probes, which can be eliminated from the isolated chromatin by mild RNase treatment.

As we become more interested in isolating regulatory proteins from specific genes, mild chemical fixation of the chromatin might be useful in preventing exchange, loss, or migration of non-histone proteins. It is unlikely that cross-linking the proteins will decrease the rate or extent of nucleoprotein hybridization, since fixation does not cause serious aggregation of the chromatin (e.g., Figure 9) and it is not expected to affect the rate-limiting step of the hybridization reaction.

Prospects of Isolation of Cellular Genes as Chromatin. To put the results of this paper into the original context of isolating specific cellular genes as chromatin, reference must be made to Figure 1, the schematic diagram of the gene isolation technique. Each step of the isolation, except prefractionation, was shown to be possible using minichromosomes. All of the steps have been tested with sea urchin embryo chromatin, with the intention of isolating the embryonic histone gene repeat from *Strongylocentrotus purpuratus* (Workman & Langmore, 1985; unpublished results). These results indicate that prefractionation of the histone genes by glycerol gradient centrifugation can enrich the early histone genes about 16-fold. Preliminary reactions of sea urchin chromatin with mercurated plasmids have given greater than 115-fold enrichments of the histone gene chromatin. Thus, both enrichment steps, performed in series, should be capable of enriching the histone genes greater than 1800-fold.

For multicopy genes (such as the histone genes of sea urchin and the 5S genes of *Xenopus*) that comprise about 0.25% of the genome, it is therefore feasible to attain purities of almost 90% after prefractionation and hybridization. Extrapolating the SV40 results to the isolation of cellular genes, we expect to isolate about 10 ng of histone gene-specific chromatin from 10 μg of sea urchin nuclei. The isolated material should contain gene-specific core histones contaminated with only about 20% nonspecific histones. This amount of chromatin would be suitable for electron microscopy, as well as electrophoretic characterization of the core histones present during regulation. ^{125}I labeling of the isolated chromatin in vitro should allow the identification of non-histone proteins present in one copy per gene. We cannot yet speculate about the fate of non-histone proteins during isolations of multicopy cellular genes. The only non-histones that we have investigated so far are the SV40 proteins VP1 and VP3, which were not lost through the multiple steps of isolation presented in this paper. There is a growing body of evidence that many regulatory proteins form complexes which are stable during many cell generations and during multiple step isolation procedures [reviewed by Brown (1984)]. Thus, we are hopeful that nucleoprotein hybridization can be used to identify the regulatory proteins associated with multicopy genes, and with future

improvements in the affinity techniques be useful in the investigation of single-copy genes of lower eukaryotes.

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