[16] Isolation, Purification, and Analysis of Specific Gene-Containing Nucleoproteins and Nucleoprotein Complexes

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Introduction

Although much has been learned about the physical structure of chromatin from intensive investigations, the role of individual nucleoproteins (NPs) with tightly bound nucleic acid in the regulation of gene expression and related chromatin enzymatic activities has for the most part received scant attention. In addition, the organization of eukaryotic genes at the level of chromatin has not been fully described and the fine structural details of chromatin beyond the description of the nucleosome spacer model are basically unknown (1–12). Moreover, important enzymatic activities, such as those involved in transcription and cellular division, have been studied only in soluble, isolated mixtures of activities and have not been examined in chromatin complexes that undoubtedly have importance *in vivo*. Thus, most of the information on transcription in mammalian systems has focused on the role of protein–DNA interactions at the DNA primary sequence level *in vitro* and not on assessing the potential role of NP–NP and NP–nucleic acid interactions.

Most conventional studies on mammalian gene regulation have employed the use of viral or other well-defined DNAs mixed with soluble enzymes or enzyme complexes to assess oligonucleotide-binding mechanics and regulation of gene expression *in vitro* (13–19). Although important to our understanding of protein–gene and protein–promoter interactions, such models may be more analogous to macromolecular interactions involved in prokaryotic gene regulation than to interactions associated with regulation of eukaryotic genes. In eukaryotic systems, transcription and other related gene-regulatory processes do not generally occur on "naked" DNA *in vivo*. To overcome this limitation, investigators have begun to study chromatin and NP interactions, such as those involved in nuclear matrix or scaffold and radial loop organization of "active" and "inactive" chromatin domains (8–10). Based on these studies, it is reasonable to hypothesize that eukaryotic genes may be anchored onto chromatin via NP macromolecular associations.

The hypothesis that NP associations and interactions are important in chromatin structure, gene regulation, and associated enzymatic activities prompted our laboratories to develop techniques to isolate, purify, and analyze NPs that contain specific, tightly bound nucleic acids and *in vitro* enzymatic activities (20–23). Our initial studies indicated that specific NPs are organized into NP precursor or subchromatin complexes. We found that these precursor NP complexes could be released from

Methods in Molecular Genetics, Volume 5

intact chromatin in nuclei by direct nuclear digestion with *Msp*I, followed by washing in the absence of chelating agents and fractionation by horizontal native, low ionic strength electrophoresis. These *Msp*I-derived precursor NP complexes consisted of specific deoxyribonucleoproteins/ribonucleoproteins that could be further fractionated into their constituent NPs by two-dimensional isofocusing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (23). By electroeluting the individual NPs from the polyacrylamide gels and removing the SDS, we could recover some of the enzymatic activity present in the NPs and NP complexes and study their interactions with specific genes or gene sequences.

Preparation of Precursor Nucleoprotein Complexes

Isolation of Nuclei

Subnuclear precursor NP complexes can be generated by direct *Msp*I restriction digestion of nuclei. In the examples presented here, nuclei isolated from chronic myelogenous leukemia cells or from clonal variants of the metastatic murine large-cell lymphoma cell line RAW117 are used as starting material (24, 25). The basic preparation and isolation scheme results in the generation of six precursor NP complex fractions, arbitrarily called fractions S1, M1, S2, M2, 0.1K, and R (Fig. 1). For example, RAW117 metastatic clonal variants grown in Petri dishes containing Dulbecco's modified Eagle's medium (DMEM) with high glucose and 5% (v/v) fetal calf serum (FCS) are transferred to glass roller bottles and grown to a maximum density of 1×10^6 cells/ml. Cells (~4–5 g) are harvested by a low-speed centrifugation for 10 min at 1000 *g* at 4° C, washed once by low-speed centrifugation at 800 *g* for 10 min in ice-cold phosphate-buffered saline (PBS; 0.15 *M* NaCl, 0.010 *M* NaH_2PO_4, pH 7.2), and resuspended in 0.010 *M* NaCl, 0.005 *M* KCl, 0.008 *M* $MgCl_2$, 0.010 *M* Tris–HCl, pH 7.4. The cells are allowed to swell on ice for 20 min. Following this incubation, the cell suspension is centrifuged at 1085 *g* for 10 min at 4° C, and the pellet is resuspended and washed in the same buffer in the presence of 0.05% nonionic detergent Nonidet P-40 (NP-40) at 4° C. The percentage of NP-40 used in this preparation varies; the more fragile the nuclei, the less NP-40 is used. More important than the percentage of detergent is the exclusion of chelating agents from all solutions, as these have a deleterious effect on precursor NP complex integrity (26). After the NP-40 buffer wash, two more washes are performed in the same buffer without NP-40, as before. Through the initial phases of preparation, nuclei prepared from chronic myelogenous leukemia cells or peripheral blood leukocytes behave in a manner similar to that of nuclei from murine RAW117 cells, except that the first two require additional steps for the lysis of contaminating red blood cells. Contaminating red blood cells are removed by separation on a Histopaque gradient

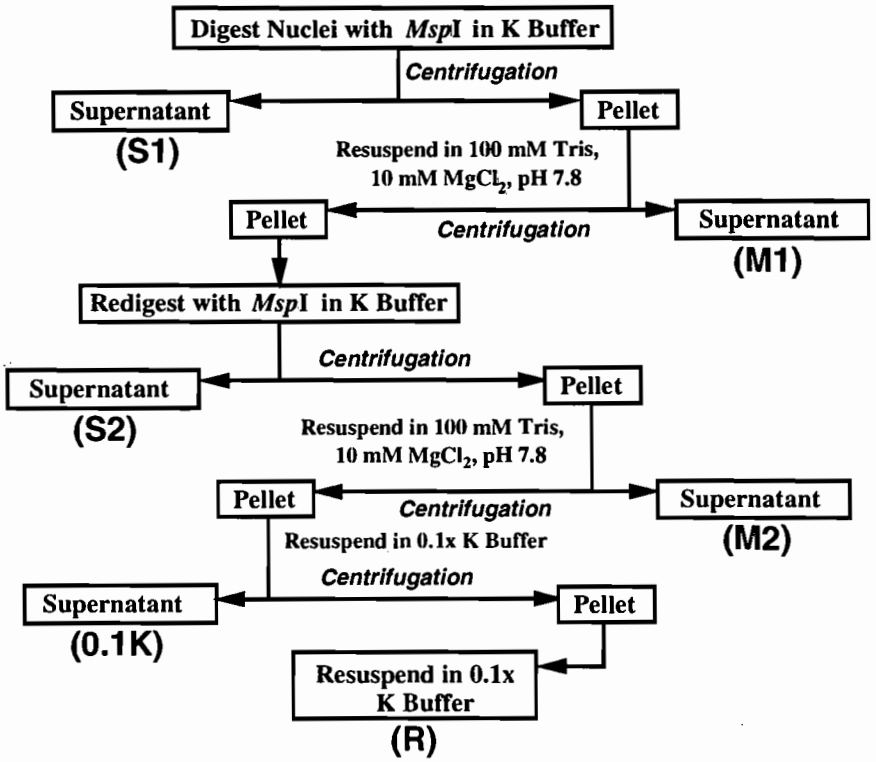

FIG. 1 Flow chart illustrating the preparative strategy for the generation of the six precursor NP complex fractions (S1, M1, S2, M2, 0.1K, and R) obtained by direct digestion of the nucleus with *Msp*I.

as follows: Whole heparinized blood (10–20 ml) is mixed with an equal volume of PBS solution, layered over 1 vol of Histopaque-1077 (Sigma, St. Louis, MO), and centrifuged for 30 min at 400 *g* at room temperature. The opaque interface containing mononuclear cells is then recovered with a pipette, mixed with an equal volume of PBS, and subsequently centrifuged for 10 min at 1000 *g*. The resultant pellet is then subjected to the identical protocol described for RAW117 cell nuclei isolation.

At each step in the preparation protocol, nuclei are monitored for morphological integrity by light phase microscopy. All buffers used in the nuclei preparation contain the protease inhibitors leupeptin (0.005 *M*) and aprotinin (50 μg/ml). The isolation of precursor NP complexes from subnuclear chromatin structures takes advantage of endogenous endonucleases; thus, PMSF is not included in the nuclei isolation because of its potential inhibition of endogenous endonuclease function.

Direct Restriction Digestion of Nuclei with MspI

Prior to restriction digestion with *Msp*I, nuclei are washed in K buffer (0.060 *M* KCl, 0.010 *M* $MgCl_2$, 10^{-5} *M* $CaCl_2$, 0.010 *M* NaCl, 0.015 *M* Tris–HCl, pH 7.5). Initial *Msp*I digestion of nuclei from RAW117 cells (1 mg/ml protein) is performed in 500-μl Eppendorf microfuge tubes at an enzyme concentration of ~1600–2000 units of *Msp*I/mg nuclei in K buffer for 2.5 hr at 37° C. Nuclei from chronic myelogenous leukemia or normal control leukocytes are more fragile, so the equivalent digestion is performed for only 1 hr under the same conditions. At the completion of the initial direct digestion with *Msp*I, the first precursor NP complex fraction, S1, corresponding to the supernatant, is generated by microcentrifugation for 1 min at 12,000 *g* at room temperature. The remaining pellet is then washed in TM buffer (0.100 *M* Tris–HCl, 0.010 *M* $MgCl_2$, pH 7.8) by resuspension, followed by vortexing and subsequent microcentrifugation for 1 min at 12,000 *g* at room temperature. The resultant supernatant from this wash step (M1) corresponds to the second precursor NP complex fraction generated by this method. At this phase in the precursor NP complex fractionation and following the microcentrifugation and resuspension in K buffer, the pellet is redigested with *Msp*I at 50% of the initial enzyme concentration for 1 hr at 37° C for RAW117 nuclei and for 15 min for nuclei from chronic myelogenous leukemia cells. The incubation times for both digestions are dependent on the inherent nature of the nuclei being treated, with more fragile nuclei subjected to direct digestion with *Msp*I for shorter periods of time. Digestion times are determined empirically by morphologic monitoring of the nuclei. The second digestion is followed by microcentrifugation at 12,000 *g* at room temperature to generate the third precursor NP complex fraction supernatant (S2). Microcentrifugation and resuspension in TM buffer are then repeated to generate the fourth precursor NP complex fraction (M2). The resultant pellet is subjected to an additional microcentrifugation and resuspension in K buffer diluted 10-fold to yield the fifth precursor NP complex fraction (0.1K). The final pellet is then resuspended in K buffer that has been diluted 10-fold by aspiration and vortexing to distribute the residual material uniformly to yield fraction R. At each step in the direct *Msp*I digestion of nuclei, aliquots of the preparation should be monitored by microscopy for morphologic integrity of the remaining nuclei.

Low Ionic Strength Electrophoresis of Nucleoprotein Precursor Complex Fractions

Native low ionic strength electrophoresis is performed using a modification of electrophoretic systems designed to fractionate ribonucleoproteins and deoxyribonucleoproteins (27, 28). Samples from precursor NP complex fractions to be fraction-

ated by native low ionic strength electrophoresis are diluted (1 : 10) with TB buffer (0.010 *M* Tris–HCl, 0.010 *M* boric acid, pH 7.8). Chelating agents are excluded from all buffers. Low ionic strength electrophoresis is performed on a minigel apparatus (Horizon, Model 200, Bethesda Research Laboratories, Gaithersburg, MD) using 1% ultrapure agarose (Bethesda Research Laboratories) in TB buffer at 75 V for approximately 1 hr. The precursor NP complex fractions are then visualized by ethidium bromide staining (1 μg/μl) under ultraviolet (UV) irradiation.

On a routine basis, our laboratories perform duplicate native low ionic strength gel electrophoresis on equivalent samples of precursor NP complex fractions: one for analytical purposes, and the other for native low ionic strength gel transfer to a solid support. Native low ionic strength gels to be transferred are first incubated for 1 hr at 37° C in 100 ml TBS solution (0.14 *M* NaCl, 0.02 *M* Tris, pH 8) containing 0.5% SDS and 100 μg/ml proteinase K. After a 45-min wash at room temperature in 1.5 *M* NaCl and 0.5 *M* NaOH, the gels are neutralized by soaking for 45 min in 1 *M* Tris, 1.5 *M* NaCl, pH 8, and transferred to Nytran by capillary transfer in 10× SSC (1× SSC = 1.5 *M* NaCl, 0.15 *M* sodium citrate). DNA is then cross-linked to the Nytran by UV irradiation (Stratalinker, Stratagene, La Jolla, CA).

Analysis of precursor NP complexes for the presence of various genes of interest is the first step in the screening of all NP complex fractions. A typical precursor NP complex native low ionic strength gel electrophoretic pattern (ethidium bromide staining) from nuclei of chronic myelogenous leukemia cells is shown in Fig. 2A, with its coincident native gel screen for *bcl-2* (a gene implicated in chromosomal breakage and apoptosis) and for *TIMP-2* (a gene encoding a tissue inhibitor of metalloproteinase) shown in Figs. 2B and 2C, respectively. Patients suffering from various forms of leukemia will possess a variable, strongly positive *bcl-2* signal among precursor NP complex fractions. These fractions are characterized by the presence of high molecular weight ethidium bromide-stained materials in all precursor NP complex fractions, whereas normal control leukocytes rarely show a strongly positive *bcl-2* signal in any particular NP fraction. Trace amounts of *bcl-2* are often found preferentially in the precursor NP complex fractions S1, M1, and S2 (data not shown). Patients who have undergone various chemotherapeutic regimens or *in vivo* treatment with a cytokine such as interferon α will for certain periods of time after treatment possess a precursor NP complex ethidium bromide staining pattern similar to that of normal leukocytes (unpublished observations, 1993). The absence of detectable ethidium bromide staining material in the NP preparations does not necessarily suggest the absence of material in precursor NP complex fractions.

The presence of *bcl-2* in the precursor NP complex fractions of chronic myelogenous leukemia cells is readily detectable and characterized by a strong hybridization signal in the S1 precursor NP complex fraction (Fig. 2B). In contrast, strong hybridization to the *TIMP-2* gene is noted in all precursor NP complex fractions obtained from chronic myelogenous leukemia cells of the same patient (Fig. 2C). We also

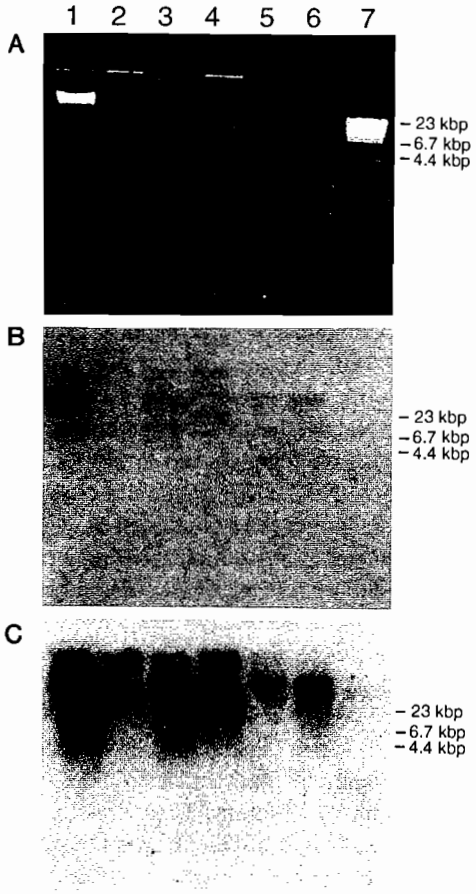

FIG. 2 Native low ionic strength electrophoresis of precursor NP complex fractions from a patient with chronic myelogenous leukemia. (A) Ethidium bromide staining pattern for precursor NP complex fractions S1, M, S2, M2, 0.1K, and R (lanes 1, 2, 3, 4, 5, and 6, respectively; lane 7, size marker). (B) Coincident screening of the native gel-resolved precursor NP complex fractions for *bcl-2* by hybridization. (C) Coincident screening of the native gel-resolved precursor NP complex fractions for *TIMP-2* gene by hybridization.

noted a similar pattern for the 72-kDa type IV collagenase gene (data not shown). Both of these genes have been implicated in tumor invasion and metastasis. The inability to detect a hybridization signal in a particular precursor NP complex fraction, however, does not preclude the presence of a low level of a particular gene in that complex.

Isolation and Purification of Individual Nucleoproteins from Precursor Nucleoprotein Complexes

Precursor NP complex fractions can be further analyzed for their NP composition, and individual NPs can be isolated and purified employing two-dimensional reducing isoelectric focusing SDS–PAGE (29, 30). The constituent NPs, particularly those with tightly associated genes, represent highly durable elements from the *Msp*I-generated subnuclear precursor NP complexes. The isolation, purification, and analysis of NPs are depicted in Fig. 3. Prior to two-dimensional reducing isoelectric focusing SDS–PAGE, equivalent precursor NP complex fractions are incubated in the presence or absence of DNase I for 1–2 hr at 37°C to further fractionate the precursor NP complex constituents into NPs containing tightly or covalently bound DNAs and RNAs. Two-dimensional reducing isoelectric focusing SDS–PAGE is performed using a duplicate gel system, with half of each gel allotted for the untreated sample and half for the sample treated with DNase I. After electrofocusing and electrophoresis in SDS, the NPs are identified by analytical silver staining. The NPs are subsequently isolated as single spots or streaks from a duplicate preparative gel stained with Coomassie blue. This is done by locating each NP constituent by its

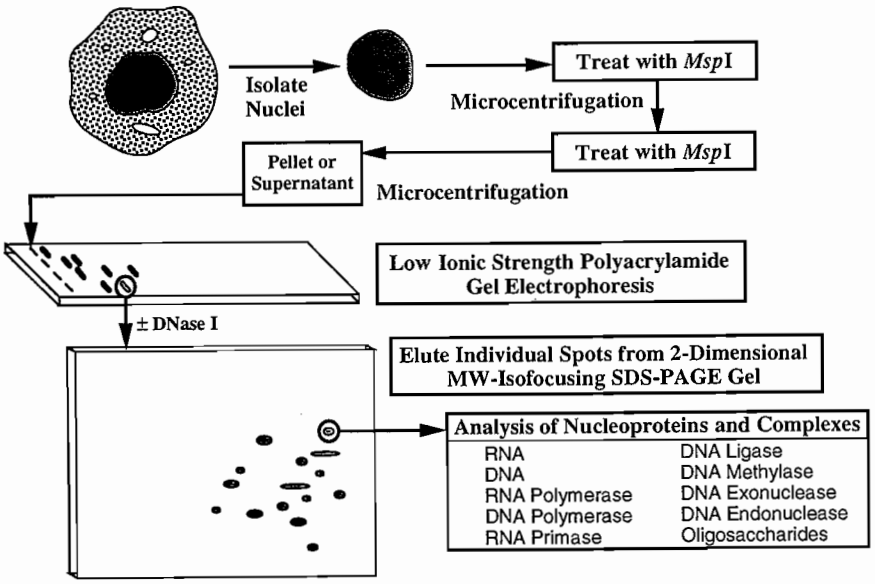

FIG. 3 Flow chart illustrating the isolation and purification of specific NPs and NP complexes and the analytical strategies employed. The flow chart outlines relationships among the nucleus, precursor NP complex fractions, and the associated NP constituents.

isoelectric point (p*I*) and apparent M_r in the silver-stained gel and then excising the NPs from the Coomassie blue-stained gel. Purification is then accomplished by electroelution from the preparative gel by transferring the excised gel pieces to a dialysis bag (M_r ~6000 exclusion) containing 500 μl of 0.025 *M* Tris, 0.190 *M* glycine, and 0.1% (w/v) SDS, and then electroeluting the NPs using a horizontal flatbed gel apparatus for approximately 1–4 hr. The resultant electroeluants are then brought to a volume of 500 μl by addition of 0.1% (w/v) bovine serum albumin (BSA) in the same buffer and subjected to extractive gel chromatography (Extracti-Gel D, Pierce, Rockford, IL) to remove SDS.

Analytical silver staining of two-dimensional reducing isoelectric focusing SDS–PAGE gels is accomplished by a standard method (31). NPs are fixed to the polyacrylamide gel matrix by immersing the gel in 40% ethanol and 10% acetic acid for 2 hr followed by equilibration (washing and rinsing the gel three times over a 30-min interval). The silver stain is prepared as follows: 1 g of $AgNO_3$ dissolved in 5 ml of distilled water is added dropwise to a solution containing 28 ml of 0.1 *M* NaOH and 2 ml of NH_4OH (28%) with constant stirring. The silver stain is then brought to a final volume by the addition of 115 ml H_2O, and the gels to be stained are then immersed in this solution for 15 min. After staining, the gels are rinsed three times in H_2O for three consecutive 20-min washes. Following the washes, the gels are immersed in 300 ml H_2O containing 75 mg of citric acid and 0.15 ml of 37% formaldehyde for silver stain development. Silver stain development is then stopped by replacing the developer solution with 10% acetic acid in 40 ml ethanol.

To distinguish NPs with an endogenous nuclear origin from those that arise from the possible exogenous contamination of the *Msp*I used in generating precursor NP complex fractions or other potential contaminants, cells may be metabolically labeled with [^{35}S]methionine. Cells to be metabolically labeled are adjusted to a concentration of 1×10^5 cells/ml in a final volume of 250 ml methionine-free DME medium (high glucose) (GIBCO, Grand Island, NY) with 5% fetal calf serum. [^{35}S]Translabel (ICN, Irvine, CA) is added to a final concentration of 5 mCi, and cells are allowed to grow for 48 hr. Cell synchronization is accomplished by a modification of the double thymidine block technique (32). A single block of 0.002 *M* thymidine is added to RAW117-H10 cell cultures 16 hr prior to cell harvesting. This approach was shown elsewhere to be effective for RAW117 cell synchronization (23). To release cells from the thymidine block, the cells are harvested by low-speed centrifugation, followed by washing and resuspension in prewarmed complete DME medium. The resultant suspension is incubated for 3 hr before the final collection of cells by low-speed centrifugation. Coincident [^{3}H]thymidine labeling indicates that at this point, the cells are undergoing peak DNA synthesis.

A typical NP analytical silver-stained two-dimensional isofocusing SDS–PAGE gel and corresponding autoradiogram for [^{35}S]labeled NPs prepared from synchronized RAW117-H10 nuclei using methods described in this chapter are shown for precursor NP complex R (Figs. 4A and 4B, respectively). To differentiate the NPs

that incorporate [^{35}S]methionine from those that do not, the silver-stained gels are treated with Enhance (New England Nuclear, Boston, MA) to improve the fluorographic procedure. Silver-stained gels treated with Enhance and dried using a conventional vacuum heat dryer are particularly prone to crack during drying. To circumvent this problem we use the Easy Breeze air-gel dryer according to instructions provided by the manufacturer (Hoefer Scientific Instruments, San Francisco, CA). After soaking the gel for 1 hr in Enhance, the gel is soaked in 35% ethanol and 2% glycerol for 2 hr and then dried using the lowest heat setting. Fluorography is then accomplished by exposing the dried gel at 80° C to Kodak (Rochester, NY) XAR-5 film in the presence of DuPont (Wilmington, DE) intensifying screens.

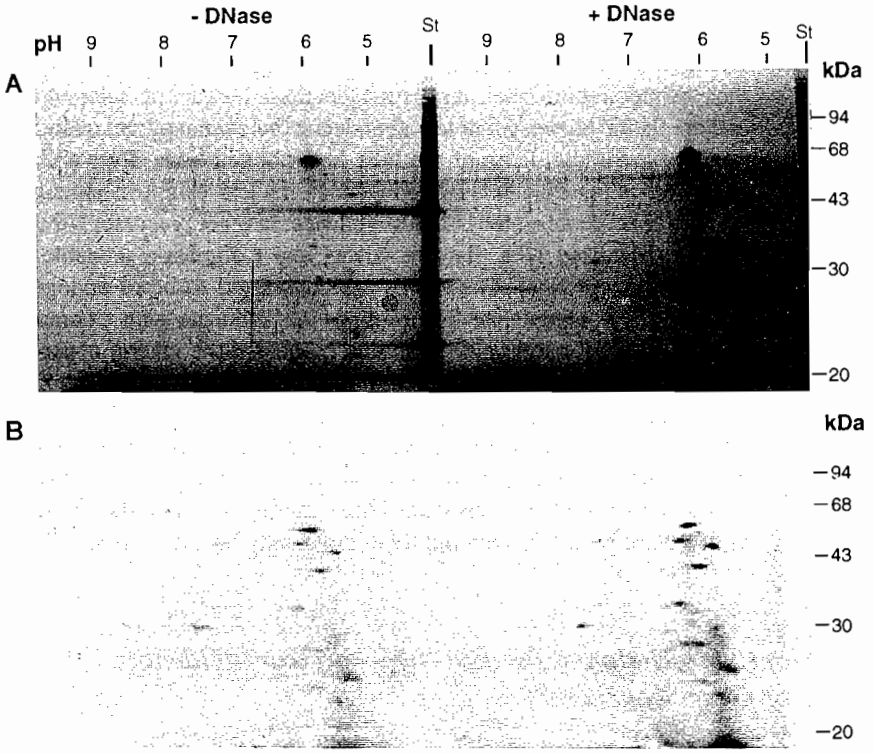

FIG. 4 (A) Characteristic analytical silver staining pattern of NPs derived from 3-hr synchronized, [^{35}S]methionine-labeled RAW117-H10 nuclei (precursor NP complex fraction R). The gel pattern was generated by two-dimensional reducing isoelectric focusing SDS–PAGE. (B) The corresponding autoradiogram of [^{35}S]methionine-labeled NPs. The labeling technique makes it possible to detect NPs in less than nanogram abundance and to discern NPs of endogenous nuclear origin from those arising from exogenous contamination.

For all preparations, aliquots of the precursor NP complexes are incubated in the presence or absence of DNase I to separate NPs with tightly or covalently bound from loosely bound DNA and RNA. By comparing NPs that incorporate [^{35}S]methionine label with those that do not or that are not coincidentally silver-stained, it is possible to categorize precisely endogenous nuclear versus exogenous origin of the individual NPs. A further delineation may then be made by comparing silver-stained versus Coomasie blue-stained NPs. We have found that the NPs possessing high relative amounts of nucleic acid often show poor silver staining. In addition, the metabolic labeling step allows us to distinguish NPs that are present in very low abundance (less than nanogram quantities) and that are not easily detectable by silver staining.

Analysis of data from one of the NP fractions isolated from RAW117-H10 nuclei at DNA peak synthesis indicates that 24 silver-stainable NPs incorporated [^{35}S]methionine label (Table I). These NPs are present in nanogram quantities and arose from the RAW117 nuclei. Ten NPs did not stain with silver but did incorporate the [^{35}S]methionine label, suggesting that these NPs are of endogenous nuclear origin and are therefore present in low abundance (below nanogram quantities). In this particular experiment, none of the NPs incorporated both silver and Coomassie blue stain, indicating that NPs of endogenous nuclear origin in precursor NP complex fraction R are represented in low abundance. In other precursor NP complex fractions we have detected NPs that are silver- and Coomassie-stainable and incorporate [^{35}S]methionine label. These NPs are considered to be of endogenous origin and relatively high abundance. The proteins that did stain with Coomassie blue did not incorporate [^{35}S]methionine label and possessed the known apparent M_r and pI of DNase I and BSA, indicating that these are exogenous proteins. Only one NP that incorporated silver stain did not correspond to BSA or DNase I, and that protein did not incorporate [^{35}S]methionine label, suggesting that this NP (with an abundance approximately in the nanogram quantity range) came from an exogenous source. The NPs that are specifically visualized only after treatment of the precursor NP complex with DNase I are NPs with tightly or covalently bound DNA and RNA.

Dot-Blot Hybridization of Purified Nucleoproteins to Identify Tightly Bound Genes or Gene Sequences

Purified NPs are screened for the presence of tightly or covalently bound genes using a dot-blot hybridization procedure originally described by Pepin *et al.* (33). Relatively few (~1%) of the purified NPs (out of ~1000) that we analyzed from a variety of cell systems possess specific tightly bound genes (in picogram quantities) (23). Using the RAW117 system we examined NPs for genes known to be expressed (*p53,* μ-chain immunoglobulin, v- or c-*abl, bcl-2,* c-H-*ras,* c-*neu,* 18S rDNA gene) or

TABLE I Constituent Nucleoproteins Isolated from R Fraction of Synchronized RAW117-H10 Cell Nuclei

		Staining reactions of NPs		
M_r ($\times 10^{-3}$)	~p*I*	Silver	Coomassie blue	Incorporation of [^{35}S]methionine
66[a,b]	6.1	+	+	−
62[a]	5.7	+	−	+
52[a]	5.2	+	−	+
46[a]	5.1	+	−	−
45[a]	5.5	+	−	+
54[a]	5.7	−	−	+
53[a]	6.7	−	−	+
38[a]	7.0	+	−	+
34[a]	7.3	+	−	+
38[a]	5.9	+	−	+
35[a,c]	5.2	+	+	−
34[a]	5.6	+	−	+
32[a]	5.8	+	−	−
30[a]	5.8	+	−	+
30[a]	5.4	+	−	+
26.5[a]	6.7	+	−	+
26[a]	5.4	+	−	+
23.5[a]	5.1	+	−	+
26.5[a]	5.0	−	−	+
17[a]	5.4	+	−	+
90	5.7	−	−	+
66[b]	6.1	+	+	−
60	5.7	+	−	+
52	5.1	+	−	+
45	5.5	−	−	+
39	7.1	+	−	+
34	7.3	+	−	+
36	5.6	+	−	+
34	5.7	+	−	+
32	5.9	+	−	+
25.5	5.6	+	−	+
26.5	5.1	−	−	+
22.5	5.2	−	−	+
14	5.1	−	−	+
15	6.6	−	−	+
15[a]	6.6	−	−	+
30[a]	6.3	+	−	+

[a] DNase I-treated NPs.
[b] Identified as BSA.
[c] Identified as DNase I.

usually not expressed (β-casein) in this cell system. Few of the two-dimensional reducing isoelectric focusing SDS–PAGE-purified NPs analyzed by this method contained more than one or two genes or parts of genes in picogram quantities (23). We found one NP in our screen that contained more than two genes or portions of gene sequences, but this NP was characterized by an ability to bind exogenous gene sequences through its protein moieties. To rule out a hybridization signal that arises from DNA–protein interactions rather than from DNA–DNA hybridization, we routinely strip the dot-blots between successive hybridization experiments with 0.1 *M* NaOH and place the dot-blot into autoradiography overnight. If the signal persists, we conclude that it is due to protein–DNA interactions rather than to DNA–DNA hybridization. In previous studies, we found that one NP dot-blot signal was attributable to protein–DNA recognition rather than to DNA–probe hybridization (23).

To identify tightly bound genes or gene sequences, aliquots (50 μl) of the purified NPs are heated to 95° C for 5 min and cooled quickly on ice. NaCl is then added to a final concentration of 2.5 *M,* and the solution is passed through a Nytran immobilization membrane (Schleicher and Schuell, Keene, NH) in a 96-well dot-blot apparatus. The membrane is then removed from the apparatus, air-dried, and baked in a vacuum oven for 2 hr at 80° C. This dot-blot method is suitable for screening large numbers of NPs purified by the methods described, but for more accurate quantitation of specific genes, we have found that a slot-blot apparatus is preferable. DNA probes used in dot-blot or slot-blot hybridization studies are labeled with [α-^{32}P]dCTP (ICN, Irvine, CA) using the Random Primed DNA labeling kit (Boehringer-Mannheim, Indianapolis, IN). Prehybridization and hybridization of the membranes are performed using Southern blot conditions (34). Posthybridization washing of the blots included the following washes: two rinses in 6× SSC-0.1% SDS for 20 min at 55° C; two rinses in 2× SSD–0.1% SDS for 20 min at 55° C; and a final rinse in 0.5%× SSC–0.1% SDS at 55° C.

A typical dot-blot template and hybridization pattern (from unsynchronized and synchronized RAW117 cells) for detection of *p53* present in NPs derived from precursor NP complex fraction R of highly metastatic RAW117-H10 nuclei as well as the dot-blot membrane prototype is shown in Figs. 5A–5C. Here we show that the *p53* gene or its sequences is contained in only one NP isolated from synchronized RAW117-H10 cells compared with eight NPs from the unsynchronized cells. The lack of detection of a gene by dot-blot or slot-blot hybridization does not necessarily imply that the gene is not present in a particular NP; it only suggests that the gene is not present in picogram amounts.

Nucleoprotein RNA Back-Hybridization Blots to Assess RNA Products

To identify the NP-associated genes present in less than picogram quantities and to correlate these NP-associated genes with a given NP's ability to initiate and support the synthesis of mRNA *in vitro,* we developed an NP RNA back-hybridization pro-

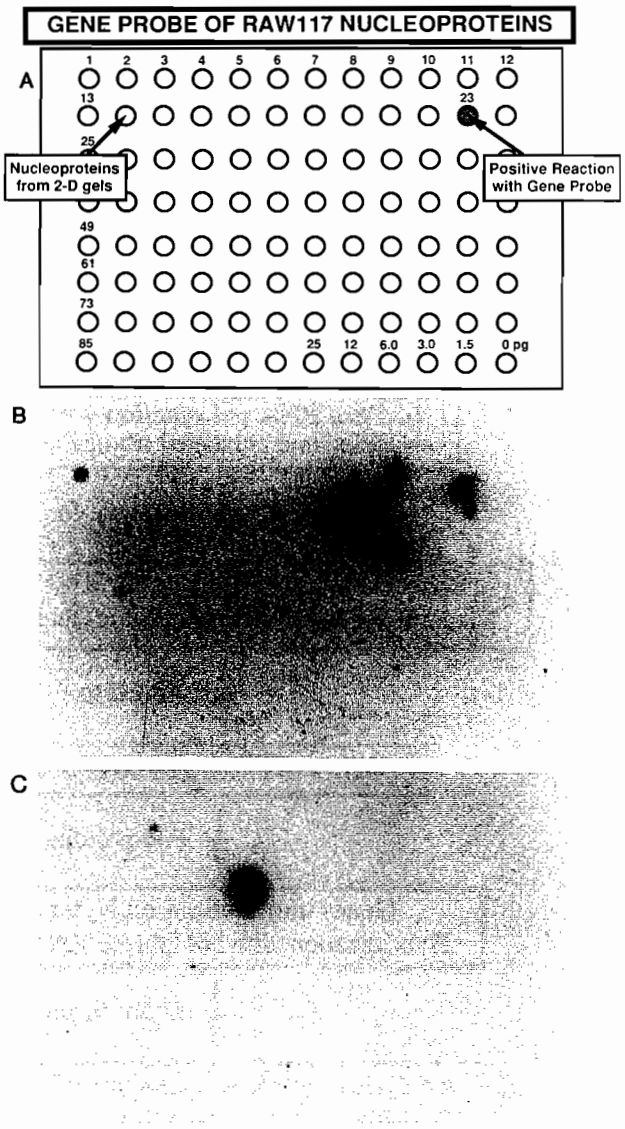

FIG. 5 Dot-blot hybridization screening for *p53*-positive NPs. (A) Dot-blot hybridization template. (B) *p53*-positive NPs isolated from unsynchronized RAW117-H10 nuclei (precursor NP complex fraction R). (C) *p53*-positive NP distribution from a 3-hr synchronized RAW117-H10 cell population (precursor NP complex fraction R).

cedure. This procedure depends on the use of one or more two-dimensional reducing isoelectric focusing SDS–PAGE-purified NPs or NP complexes that contain RNA polymerase activity in an *in vitro* RNA synthesis assay designed to generate

NP-associated mRNAs from endogenous DNAs. Individual NPs or combinations of NPs are used to generate *in vitro* synthesized mRNAs, which after further purification and end-labeling, are then used to probe back to specific genes immobilized on strips of Nytran. A flow chart describing the back-hybridization scheme is shown in Fig. 6. Nytran strips with immobilized genes of interest are hybridized with purified, end-labeled RNAs from particular NP combinations and prepared as follows. Unlabeled cDNA probes (~25 pg) are immobilized onto Nytran strips according to the method described by Pepin *et al.* (33). We have found that either 8 or 12 cDNAs, respectively, can be conveniently analyzed by this procedure, depending on whether a vertical or horizontal sequential blotting arrangement is chosen. Nytran strips containing immobilized cDNAs of interest are then subjected to vacuum baking or to

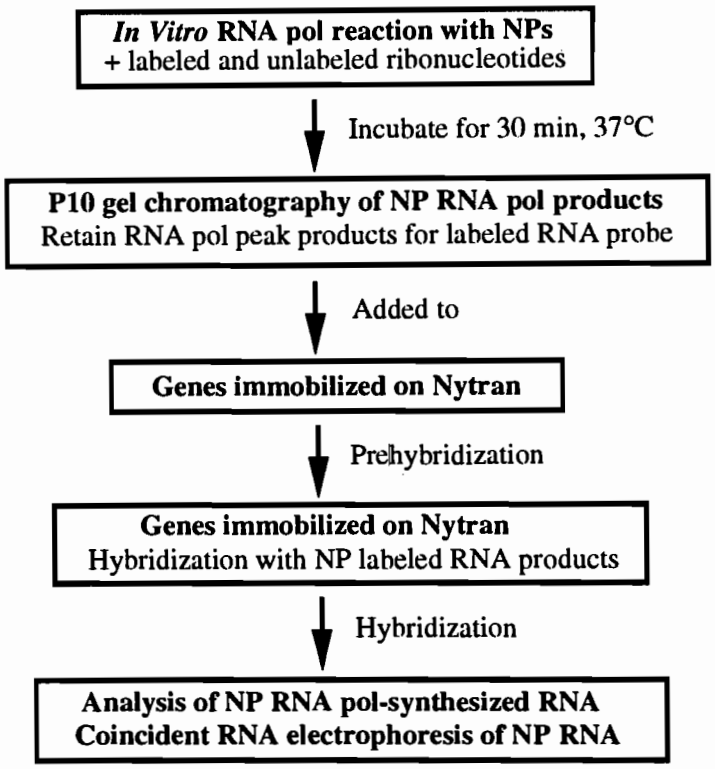

FIG. 6 Flow chart illustrating the RNA back-hybridization procedure and coincident RNA analysis by RNA glyoxyl gel electrophoresis. NPs are used to generate *in vitro* synthesized RNAs, which are used as probes to hybridize to Nytran-immobilized cDNAs for *p53,* v- or c-*abl, μ-chain* gene, and other genes as shown. Coincident glyoxyl RNA gel electrophoresis is used to assess the molecular weight distribution and apparent sizes of the *in vitro* synthesized RNA probes.

UV cross-linking and placed in a bag and heat-sealed between adjacent strips. The strips are prehybridized in 5 × SSC, with 22 μg/ml salmon sperm DNA, 0.01% SDS, 5 × Denhardt's solution, and 50% formamide at 42° C.

The RNA transcripts are synthesized at 37° C during a 0.5–3-hr *in vitro* reaction (total volume ~30 μl) in a 0.1 *M* Tris buffer containing a final concentration of 0.01 *M* $MgCl_2$, pH 7.5. Reactions are performed in the presence or absence of actinomycin D (RNA primase inhibitor). For reactions that include actinomycin D, the inhibitor is added at a concentration of 20-times (w/w) excess inhibitor. [α-^{32}P]GTP is added to the reaction (~1–5 μCi) containing final concentrations of ~0.1 ng of the unlabeled ribonucleotides rGTP, rATP, and rCTP. The synthesized RNA transcripts are then further purified by Excellulose (Pierce) chromatography, removing unreacted [α-^{32}P]GTP from the *in vitro* RNA synthesis reactions. The column void-volume fraction is adjusted to hybridization buffer conditions as described in previous sections, and the purified RNAs are subsequently used as back-hybridization probes to the cDNAs of genes of interest immobilized to the Nytran solid support. Hybridization is performed overnight at 42° C. The washing conditions are identical to those described for the dot-blot assays, except that the final wash is in 2 × SSC at 55° C.

Characteristic back-hybridization patterns using NP-synthesized RNA as probe to hybridize "back" to Nytran-immobilized cDNAs corresponding to genes of interest in our studies have been found for various NPs as well as for combinations of NPs. For example, the pattern found for a specific mixture of NPs of apparent M_r of ~34,000, ~35,000, and ~29,000 with p*I* values of ~5.5, ~5.2, and ~5.2, respectively, from RAW117-H10 nuclei is shown in Fig. 7A. The coincident RNA glyoxyl gel indicating that the RNA probe from this NP combination is composed of high molecular weight RNAs is shown in Fig. 7B. In the presence of actinomycin D, the NPs fail to synthesize RNA efficiently, suggesting that the NPs possess RNA primase capability (Fig. 7C) (23). If the NP combination is varied by substituting the NP of apparent M_r ~29,000, p*I* ~5.2 with an NP of apparent M_r ~19,000, p*I* ~8.0, an unknown RNA is synthesized. In this case, NPs containing μ-chain immunoglobulin gene and v- or c-*abl* DNA sequences (in picogram amounts from the dot-blot experiments described above) produce RNA transcripts to *p53* and μ-chain immunoglobulin genes but not to *abl*. Although *p53* DNA is present in one of the NPs used in the *in vitro* transcription reaction, it is present in amounts below the level of detection by the standard dot-blot procedure (23). This suggests that the mere presence of a particular gene or gene sequence in an NP does not ensure the synthesis of its RNA. Thus, specific macromolecular associations determined by the nature of an NP's RNAs, DNAs, and proteins (polymerases or polymerase-related molecules) probably determine its transcriptional competence. Subtle variations in the synthesis of RNAs can occur, and these may be dependent on particular interactions between NPs in the transcriptional complex which, in turn, can influence the nature of the RNA products synthesized *in vitro* (23, 29).

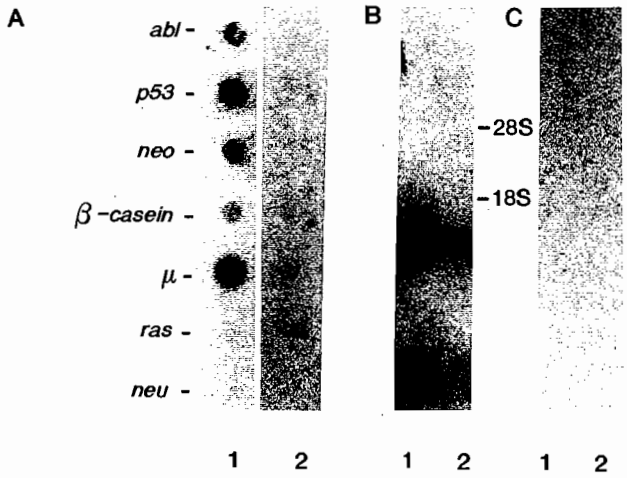

FIG. 7 RNA back-hybridization studies with coincident RNA glyoxyl gel electrophoresis. (A) RNA products synthesized *in vitro* by a combination of NPs of apparent M_r of ~34,000, ~35,000, and ~29,000 with p*I* values of ~5.5, ~5.2, and ~5.2, respectively, isolated from unsynchronized RAW117-H10 nuclei, were used as RNA back-hybridization probes to c-DNAs immobilized on Nytran. Lane 1, this NP combination, which by dot-blot hybridization was shown previously to contain picogram amounts of *μ-chain,* v- or c-*abl,* and *p53* genes, produces RNA transcripts against *p53* and *μ-chain* gene but not against c-*abl.* Lane 2, if the NP of apparent M_r ~29,000, p*I* ~5.2 is replaced by an NP of apparent M_r ~19,000, p*I* ~8, an unknown RNA is synthesized and a hybridization signal is not detected. (B) The corresponding NP *in vitro* synthesized RNAs containing [α-^{32}P]GTP label to be used as probes are resolved by glyoxyl gel electrophoresis and visualized by ethidium bromide staining. Lane 1, RNA products from an NP combination of apparent M_r of ~34,000, ~35,000, and ~29,000 and p*I* values of ~5.5, ~5.2, and ~5.2, respectively. Lane 2, RNA products from a similar combination but an NP of apparent M_r ~19,000, p*I* ~8 has been substituted for the NP of apparent M_r ~29,000, p*I* ~5.2. (C) The corresponding experiment as in (B), except that actinomycin D is included in the NP *in vitro* RNA synthesis reaction.

Discussion

We have developed procedures to purify and analyze NPs from certain chromatin domains of the eukaryotic nucleus. The main advantage of these procedures is that two-dimensional reducing isoelectric-focusing SDS–PAGE can be used to purify NPs in a highly stable state, retaining enzymatic activity and bound nucleic acids. We have found that the NPs contain tightly bound DNA and RNA and that the presence of these tightly bound nucleic acids may protect the NPs from denaturation during isolation procedures that utilize denaturing detergents such as NP-40 and SDS.

Some of the isolated NPs are macromolecules that contain specific genes in association with polymerase and polymerase-related enzymatic activities. Using a murine large-cell lymphoma cell system and human leukemia cells, we have been able to isolate specific NPs with tightly bound oncogenes and suppressor genes or their gene sequences. The role of these NPs and their associated genes is unknown, but we hypothesize that the NPs may play a role in gene regulation or other important cellular processes. For example, we previously found that the presence of certain NPs could change the types of RNA transcripts synthesized by combinations of various NPs (23).

At this phase in our studies, we do not know precisely how the isolated NPs relate to chromatin nucleosome-spacer structures, but we have observed that histone-like proteins are often associated with the NPs. In addition, the sizes we have estimated for the transcriptionally active NPs in SDS–PAGE are quite low compared with known M_r of characterized RNA polymerases. If, however, the NPs are retreated with DNase I and are rerun in a reducing SDS–PAGE system, their corresponding molecular weights are three to five times higher than those found for the untreated NPs, suggesting that the NPs are compacted by a nucleic acid shell. We have hypothesized that the gene regulatory processes involving the NPs may represent another possible level of eukaryotic gene regulation. Although the relationship of the NPs with various soluble transcription factors is unknown, the NPs may represent subsets of known transcription factors. Indeed, we have found that the transcription factor ISGF3, which is involved in interferon gene regulation, may be represented by a subset of NPs (unpublished observations, 1993). Additional research will be necessary to ascertain the role of NPs in gene regulatory processes *in vivo*.

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