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Nucleoproteins Derived From Subnuclear RNA Polymerase Complexes of Metastatic Large-Cell Lymphoma Cells Possess Transcription Activities and Regulatory Properties In Vitro

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Abstract Intact nuclei derived from poorly or highly liver-metastatic murine large-cell lymphoma cell line RAW117 were digested to discrete subchromatin deoxyribonucleoprotein/ribonucleoprotein (DNP/RNP) complexes with *Msp*-I. The DNP/RNP complexes were composed of DNP/RNPs which were derived from the DNP/RNP complexes by incubation in the presence or absence of DNase-I and subsequent isolation by two-dimensional [isoelectric focusing/sodium dodecylsulfate (SDS)] polyacrylamide gel electrophoresis (PAGE), electroelution from the gel, and removal of SDS. Approximately 450 DNP/RNPs in the two-dimensional gels corresponding to discrete spots or in some cases streaks were analyzed for the presence of v-*abl*, *p53*, c-*neu*, c-H-*ras*, β-casein, 18s rDNA, and μ-chain immunoglobulin genes using a hybridization technique. Ten DNP/RNP complexes contained tightly associated *p53* DNA, whereas six contained c- or v-*abl*, four contained μ-chain gene, two contained c-H-*ras*, one contained dot-blot β-casein, two contained 18s rDNA, and c-*neu* was found in one of the DNP/RNPs. The DNP/RNPs were also analyzed for in vitro RNA polymerase and primase activities. To assess the potential transcription abilities of the isolated DNP/RNPs, individual DNP/RNPs or DNP/RNP mixtures (reconstituted after SDS-PAGE separation) were examined for RNA polymerase initiation and synthesis. When RNA products were formed, these were purified by extracellulose chromatography and used as back-hybridization probes for the genes of interest. The RNA products were also analyzed by RNA gel electrophoresis. RNA formation was inhibitable by actinomycin D, and the RNAs formed ranged in size from ~80 kbp to ~1 kbp. By mixing various DNP/RNP complexes together, different patterns of RNA synthesis were found. For example, one DNP/RNP of M_r ~140,000, isoelectric point (pI) ~5.8 synthesized a high molecular weight RNA in vitro that hybridized with β-casein cDNA, but β-casein is not expressed in RAW117 cells, suggesting that the silencing of the β-casein gene was negated by isolation of the DNP/RNP. Mixing this DNP/RNP with two other specific DNP/RNPs again inhibited the synthesis of β-casein RNA, suggesting that interactions between DNP/RNP complexes can result in differential RNA expression or regulation of RNA polymerases in vitro.

Key words: polymerase, oncogene, hybridization, RNA synthesis, enzyme complexes, dot-blot hybridization

The mechanisms involved in eukaryotic RNA transcriptional control have been intensely investigated [Simpson, 1982; Dynan and Tjian, 1983; Samuels et al., 1982; Manley, 1983; Reeder and Sollner-Webb, 1990; Sawadogo and Sentenac, 1990]. Although considerable progress has been made in understanding the role of primary DNA sequence motifs and the interactions of key soluble factors in regulating transcription, the contribution of deoxyribonucleoprotein/ribonucleoproteins (DNP/RNPs) and higher level chromatin domains in such regulation are poorly understood. Hypersensitive restriction enzyme cleavage sites have been implicated in delineating active chromatin domains, and chromatin macromolecular structure has been divided into higher order domains constituting "active" and "inactive" DNA loop-containing chromatin regions [Finch and Klug, 1976; Thoma et al., 1979; Marsden and Laemmli, 1979; Lebkowski

Abbreviations used: BSA, bovine serum albumin; DNP/RNP, deoxyribonucleoprotein/ribonucleoprotein; PAGE, polyacrylamide gel electrophoresis; PI, isoelectric point; pol, polymerase; 2-D, two dimensional; 1-D one dimensional; SDS, sodium dodecylsulfate.

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and Laemmli, 1982; Emerson and Felsenfeld, 1983; McGhee et al., 1983; Nickol and Felsenfeld, 1983; Ramsay et al., 1984].

Most DNA-RNA-protein interaction studies have examined chromatin structure at large and have not been principally directed at elucidating possible mechanisms that could be involved in the regulation of eukaryotic transcription. In contrast, classical transcription studies have used nuclear and whole cell-free extracts and, in some cases, chromatographically purified components, but only rarely have DNP/RNP complexes been isolated from nuclei and analyzed for in vitro transcription capability [Coppola and Luse, 1984; Miller et al., 1985; Learned et al., 1985; Rosenberg-Nicolson and Nicolson, 1992]. Unfortunately, the potential relationships between the polymerases, DNA-binding factors, and chromatin structure have not been adequately assessed using DNP/RNPs.

Here we describe the isolation and purification of novel DNP/RNPs that comprise DNP/RNP complexes (repliscriptons) released from intact nuclei by *Msp*-I treatment in mild detergent solutions [Rosenberg, 1986, 1987a,b; Rosenberg-Nicolson and Nicolson, 1992]. Our rationale for these studies was to further characterize and assess the constituents of the repliscripton DNP/RNP multienzyme complexes that were capable of in vitro DNA synthesis using endogenous substrates [Rosenberg-Nicolson and Nicolson, 1992]. The DNP/RNP complexes possessed RNA polymerase (pol) activity as well, and we speculated that they could be part of the control apparatus of the eukaryotic replication machinery. The basis for studies presented in this report was our hypothesis that individual DNP/RNP components of the precursor DNP/RNP complexes should be highly durable. The DNP/RNPs analyzed here were shown to retain relatively complex in vitro functions after an isolation procedure that involved two-dimensional (2-D) isofocusing/sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by the removal of SDS. We have focused on some of the DNP/RNPs from the DNP/RNP complex precursors that contain DNA templates and specific RNAs at detectable levels that remained bound to the DNP/RNPs after treatment with DNase-I, SDS, urea, and high temperature. Some of the isolated DNP/RNPs retained their RNA pol initiation and elongation capabilities after the removal of detergent by extractive gel chromatography. Because these DNP/RNPs had apparent M_rs that did not correspond to the subunit sizes of the known RNA pols [Buhler et al., 1986], they could be a novel subset or class of tightly bound RNA pols.

MATERIALS AND METHODS

Isolation of Subnuclear DNP/RNP Complexes

Subnuclear DNP/RNP complexes were released by direct *Msp*-I restriction digestion of nuclei of the highly metastatic murine large-cell lymphoma RAW117-H10 line or its poorly metastatic parental counterpart (RAW117-P) [Brunson and Nicolson, 1978] and purified using a modification of the low ionic strength gel system originally developed for fractionation of nucleosomes and nucleosomal oligosomes [Igo-Kemenes and Zachau, 1977]. Nuclei from the various cell lines were prepared as follows: Cells ($\sim$4–5 g) were washed once by low speed centrifugation at 800*g* for 10 min in ice-cold 0.010 M NaCl, 0.005 M KCl, 8 mM $MgCl_2$, 0.88 M sucrose. The resultant pellet was resuspended in ice-cold 0.010 M NaCl, 0.015 M $MgCl_2$, 0.010 M Tris/HCl, pH 7.4, and allowed to swell on ice for 20 min. Following this incubation, the pellet was centrifuged at 1,085*g* for 10 min and washed in the same buffer in the presence of 0.005% NP-40. At this phase of the preparation, two more wash steps in buffer were performed with centrifugation, as before. The following protease inhibitors were included in all buffers and solutions: 5 mM phenylmethyl sulfonyl fluoride and 50 μg/ml aprotinin. Prior to restriction digestion with *Msp*-I, the nuclei were washed in the following buffer (K buffer) [Labhart and Koller, 1982]: 0.060 M KCl, 0.010 M $MgCl_2$, 10^{-5} M $CaCl_2$, 0.015 M Tris/HCl, 0.015 M NaCl, pH 7.5. The morphological integrity of the nuclei preparation was monitored at each step by phase microscopy.

Nuclei (1 mg/ml protein) were digested with *Msp*-I (1,600 units, Bethesda Research Laboratories, Gaithersburg, MD) in 500 μl Eppendorf microfuge tubes for 2.5 h at 37°C, and DNP/RNPs were fractionated as shown in Figure 1. Typically, we found that DNP/RNP complex yields were optimized by performing 6–8 minidigestions with $\sim$180–200 μl volumes. After the first treatment with *Msp*-I, digests were microfuged at maximum speed for 30 s, and the supernatant (S1) was decanted and retained for further analysis. The remaining pellet was then washed with 500 μl of 0.010 M $MgCl_2$, 0.010 M Tris/HCl, pH 7.5 (M buffer). This was accom-

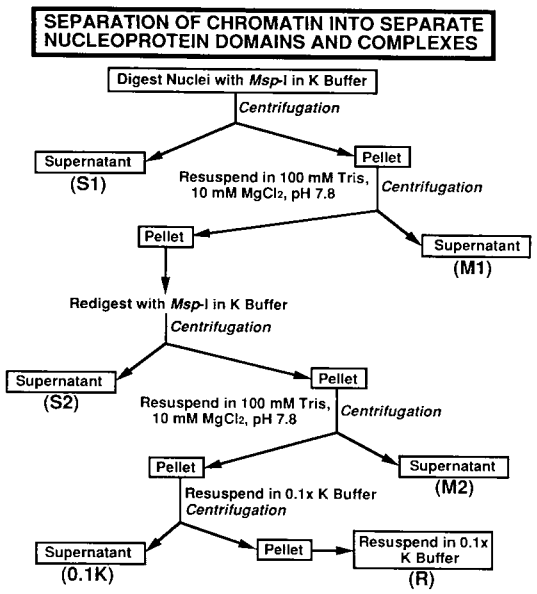


Fig. 1. Flow chart summarizing DNP/RNP fractionation from RAW117 nuclei. DNP/RNPs were released from purified RAW117 nuclei by direct digestion with *Msp*-I followed by microcentrifugation and washing. The DNP/RNP complexes were separated into fractions S1, M1, S2, M2, 0.1K, and R.

plished by resuspending the pellet by gentle vortexing and aspiration, incubating the mixture for 5–20 min at room temperature, and microfuging as before. The resultant supernatant (M1) was decanted and retained for further analysis. The pellet was then retreated with *Msp*-I under the same conditions as before, but for only 1 h. The redigested pellet was microfuged for 15 s at maximum speed, and the resultant supernatant (S2) was decanted and retained for study. The pellet was again washed in M buffer as before and then microfuged in the same manner. The resultant supernatant in M buffer (M2) was decanted and retained for further study. The remaining pellet was then washed in 0.1K (1:10 diluted)/K buffer and microfuged as before. The final supernatant (0.1K) was decanted and retained for study, and the final pellet (R) was resuspended in 0.1K buffer and also retained for analysis.

All six fractions of various subnuclear DNP/RNP complexes were then analyzed by native DNP/RNP low ionic strength gel electrophoresis. Samples for native DNP/RNP low ionic strength gel electrophoresis were prepared by diluting with a sample buffer of 0.010 M Tris/HCl, 0.010 M boric acid, pH 7.8 (TB buffer). Low ionic strength electrophoresis was performed on a minigel apparatus (Horizon™, Model 200, Bethesda Research Laboratories) using 1% ultrapure agarose (Bethesda Research Laboratories) in TB buffer at 75 mV for roughly 1 h. Chelating agents were omitted, and *Msp*-I released DNP/RNPs were visualized by ethidium bromide staining (1 μg/μl) under ultraviolet (UV) irradiation.

Isolation of Subcomplex DNP/RNPs

Approximately 450 DNP/RNPs which comprise the *Msp*-I released DNP/RNP precursor complexes were isolated from nuclei by the following method: DNP/RNPs from all six subnuclear DNP/RNP complex fractions (S1, M1, S2, M2, 0.1K, R) were analyzed using 2-D reducing isofocusing/SDS-PAGE. The protein components were identified by coincident analytical silver staining and Coomassie blue staining. Each DNP/RNP was located definitively by isoelectric point (pI) and apparent M_r, and after careful measurement of the DNP/RNP spots' (in some cases streaks) position in the gel, they were excised from the Coomassie-stained gel. The excised gel pieces were transferred to a dialysis bag containing 500 μl of 0.025 M Tris, 0.190 M glycine, and 0.1% SDS and subjected to electroelution using a horizontal flatbed gel apparatus for approximately 1–4 h. The electroeluants were then brought to a volume of 500 μl with addition of 0.1% bovine serum albumin (BSA) in the same buffer and were subjected to extractive gel chromatography (Extracti-Gel D™, Pierce, Rockford, IL) to remove SDS. DNP/RNP recoveries were 25–75% as demonstrated by the recovery of [^{35}S]methionine-labeled proteins. Samples were stored at 4°C.

In vitro RNA pol and primase assays were performed on the DNP/RNP samples after removal of SDS by conventional procedures [Waltschewa, 1980; Heberlein et al., 1985].

Dot-Blot Hybridization

The DNP/RNPs were analyzed for the presence of various genes using a dot-blot hybridization technique performed according to Pepin et al. [1990]. Aliquots (50 μl) containing the purified DNP/RNPs of interest were heated to 95°C for 5 min and cooled quickly on ice. NaCl was added to a final concentration of 2.5 M, and the solution was passed through a Nytran immobilization membrane (Schleicher and Schuell, Keene, NH) in a 96 well dot-blot apparatus. The membrane was removed from the apparatus, air dried, and vacuum oven baked for 2 h at 80°C. Prehybridization and hybridization of the mem-

branes were performed as previously described using Southern blot conditions [Maniatis et al., 1982]. DNA probes were labeled with [α^{32}P]dCTP (ICN, Irvine, CA) using the Random Primed DNA Labeling Kit™ (Boehringer Mannheim Biochemicals, Indianapolis, IN). Posthybridization washing of the blots included the following washes: twice in 6× SSC–0.1% SDS for 20 min at 55°C; twice in 2× SSC–0.1% SDS for 20 min at 55°C; and a final rinse in 0.5× SSC–0.1% SDS at 55°C. The blots were stripped of residual hybridization probes between successive hybridization by washing with 0.1 N NaOH for 5 min, and they were rinsed twice in deionized H_2O, followed by a final rinse in 0.5× SSC.

The following probes were used on the immobilized DNP/RNPs: a 1.5 kbp *Bgl*II fragment of the Ableson murine leukemia (*abl*) cDNA [Srinivasan et al., 1981], a 1.8 kbp *Sal*I-*Eco*RI fragment of murine *p53* cDNA [Harlow et al., 1985], a 2.3 kbp *Bam*HI-*Hin*DIII fragment of the pSV2*neo* plasmid vector (negative control) [Southern and Berg, 1982], a 430 bp (or 370 bp) *Pst*I fragment of mouse β-casein cDNA [Gupta et al., 1982], a 700 bp *Hin*DIII-*Eco*RI fragment of murine μ-chain immunoglobulin cDNA (generously provided by Prof. J.D. Capra, University of Texas Southwestern Medical Center, Dallas), a 2.9 kbp *Sst*I fragment of the human c-H-*ras* cDNA [Tainsky et al., 1987], and a 0.8 kbp *Bam*HI fragment of rat c-*neu* cDNA [Bargmann et al., 1986]. For a positive (quantitative) control, whole cell RAW117-H10 DNA was isolated and purified by standard phenol extraction, ethanol precipitation, and washing using procedures described above, followed by digestion with *Msp*-I.

RNA Back-Hybridization and DNP/RNP Reconstitution

The unlabeled gene probes listed in the previous section were immobilized to Nytran as described earlier using 25 pg of the appropriate DNA. A total of 8 gene probes were loaded on the vertical rows of a 96 well spot blot apparatus, such that a strip of Nytran could be cut off with the 8 probes arrayed from top to bottom in a consistent order. After vacuum baking, the strips were prehybridized in 5× SSC, 22 μg/ml salmon sperm DNA, 0.01% SDS, 5× Denhardt's solution, and 50% formamide at 42°C.

DNP/RNP-derived in vitro RNA transcripts synthesized in the presence or absence of actinomycin D by individual DNP/RNPs or mixtures of specific DNP/RNP "reconstitutes" were purified and used as back-hybridization probes to the eight immobilized genes of interest. Unreacted [α^{32}P]GTP was removed from the in vitro synthesized RNA transcript reactions by Extracellulose™ desalting chromatographic columns (Pierce). The column void volume fraction was adjusted to hybridization buffer conditions as described in the previous section, and hybridization occurred overnight at 42°C. The washing conditions were identical to those described for the dot-blot analyses and hybridization, with the exception that the final wash used 2× SSC at 55°C.

In Vitro RNA Pol and Primase

In vitro transcription studies were performed using the 2-D isofocusing/SDS-PAGE-purified DNP/RNPs as substrate with their tightly bound DNA sequences serving as template and associated proteins serving as the RNA pol enzymes and cofactors. These studies were carried out with all 450 DNP/RNPs isolated by the 2-D isofocusing/SDS-PAGE, followed by excision, elution, and removal of SDS. In this report, however, we have focused on the 72 DNP/RNPs shown to be contained in particular DNP/RNP complexes that hybridize with the genes of interest. Reconstituted DNP/RNP mixtures using a minimum of three isolated DNP/RNPs were also assessed for in vitro RNA pol activity. Unlabeled ribonucleotides (rUTP, rATP, rCTP, and rGTP) were added to the assay to a final concentration of 1 ng per reaction in buffer at a final concentration of 30 mM Tris/HCl, pH 7.5, with 3 mM $MgCl_2$, 1 μg/μl BSA, and 5 μCi [α^{32}P]GTP or [α^{32}P]UTP (Amersham, Arlington Heights, IL). Reactions continued for 30–60 min at 37°C. The presence of an RNA pol II-type activity was determined by performing the in vitro reactions in the presence of 200× (w/w) excess α-amanitin, which affects RNA pols II and III but not pol I [Heberlein et al., 1985]. RNA primase activity was assessed using 20× (w/w) excess actinomycin D to inhibit RNA primase [Waltschewa, 1980]. Enzyme products were captured using a standard filter-binding assay, in which the incubated reactions were spotted onto GF/C Whatmann filter discs (Whatmann, Hillsboro, OR) and washed with ice-cold 10% trichloroacetic acid. Those macromolecules incorporating the radiolabel were retained on the filter disc after extensive washing. The filter discs were counted in Safety-Solve (Research Products In-

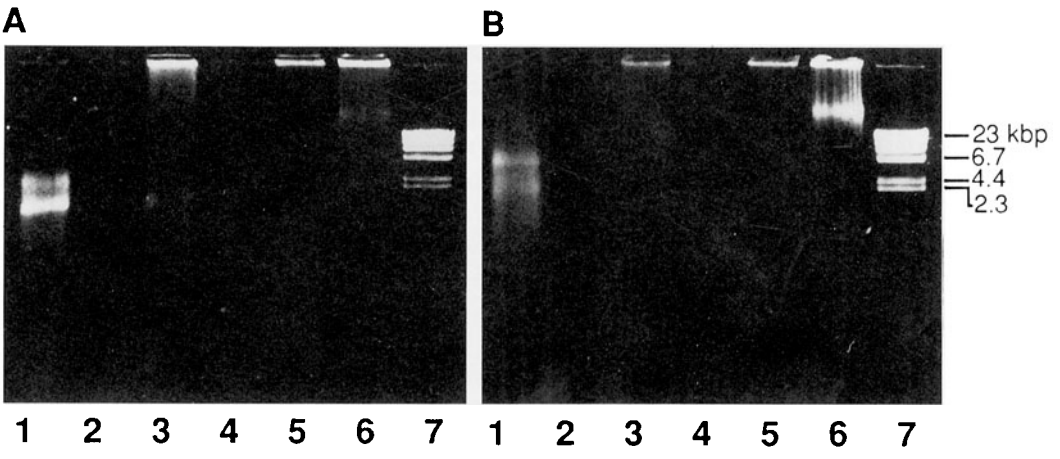


Fig. 2. Native low ionic strength electrophoresis of DNP/RNP complexes. DNP/RNP complexes were subjected to fractionation using native low ionic strength electrophoresis. **A** and **B** show patterns obtained for DNP/RNP complexes isolated from RAW117-H10 and -P cell lines, respectively. **Lane 1,** fraction S1; **lane 2,** fraction M1; **lane 3,** fraction S2; **lane 4,** fraction M2; **lane 5,** fraction 0.1K; **lane 6,** fraction R; **lane 7,** *Hin*DIII-digested λ marker DNA.

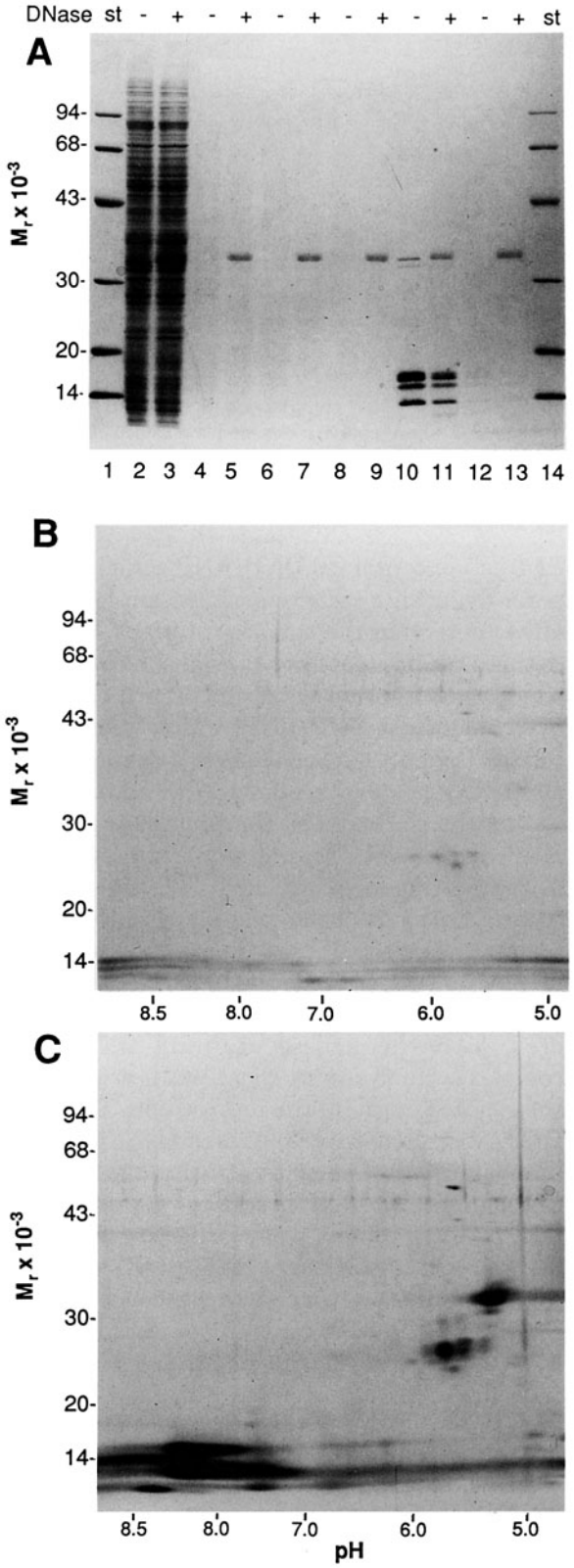
ternational Corporation, Mt. Prospect, IL) using a Beckmann scintillation counter.

For back-hybridization studies using particular DNP/RNPs or reconstituted DNP/RNP mixtures, the in vitro RNA pol products were purified by extracellulose chromatography and used as probes for the genes of interest. Analyses of the in vitro RNA products were accomplished by performing a modification of RNA gel electrophoresis using a formamide buffer at a concentration of 0.37 M as described by Maniatis et al. [1982].

RESULTS

DNP/RNP Complex Isolation and Fractionation

The precursor DNP/RNP complex fractionation procedure generated six major DNP/RNP complex categories: S1, M1, S2, M2, 0.1K, and R (see Fig. 1). After native low ionic strength electrophoresis to resolve the DNP/RNP complexes from both the highly metastatic RAW117-H10 and poorly metastic RAW117-P cell lines (Fig. 2), individual DNP/RNPs in the complexes were isolated and purified using 2-D isofocusing/SDS-PAGE (Fig. 3B,C, Tables I, II). Initially, the purity of the DNP/RNP complex fractions was assessed by one-dimensional (1-D) SDS-PAGE in the presence or absence of treatment with DNase-I (Fig. 3A). A typical pattern for RAW117-H10 DNP/RNP complex fraction R (H10-R) from unsynchronized cells is shown (Fig. 3B,C). DNP/RNPs that isofocused to apparently identical positions before and after DNase-I treatment were *not* assumed to be identical. The S1 fraction contained DNP/RNP complexes apparently lacking (or at concentration levels too low to detect) in the majority of genes screened in these studies (data not shown). The exception was a weak hybridization signal for β-casein in one constituent DNP/RNP, which was characterized by the presence of several proteins. The DNP/RNP precursor complex fractions that possessed the majority of gene-containing DNP/RNPs in our screen were fractions 0.1K and R. These two fractions contained relatively few nonhistone DNP/RNPs that were apparently tightly bound to nucleic acid, as determined by treatment with DNase-I followed by gel electrophoresis. The low level of nonhistone proteins associated with the gene-containing DNP/RNP complexes analyzed in these studies indicates the relative purity of the preparation. To assess DNP/RNP pI and apparent M_r and to determine any potential effect of the DNase-I treatment, equivalent DNP/RNP complexes from the H10 DNP/RNP fractions were either incubated in the presence or absence of DNase-I for 30 min at 37°C and then subjected to isofocusing/SDS-PAGE followed by silver staining. A representative 2-D pattern corresponding to H10-R fraction from an unsynchronized RAW117 cell population is shown in Figure 3B (before DNase-I) and Figure 3C (DNase-I treated). The 2-D pattern is characterized by a cluster of DNP/RNPs of pI ~ 5–6 in the DNase-I-treated sample with some low abundance DNP/RNPs distributed over a basic pI range and apparent M_r range ~ 11,000–14,000. The 2-D pattern for frac-



tion P-R is similar but with subtle differences in the nonhistone proteins (data not shown). We found from the 2-D electrophoresis experiment that it was essential to treat the sample with DNase-I in order to visualize many of those proteins in the DNP/RNP complexes by SDS-PAGE. That there were relatively few DNP/RNPs visualized by 2-D electrophoresis substantiates the relative purity of the precursor DNP/RNP complexes.

The 2-D electrophoresis pattern of the R fraction from *Msp*-I-digested RAW117-H10 nuclei was defined by the persistence of a low abundance triad of DNP/RNPs ranging in size from apparent M_r ~ 18,000–19,000, with corresponding pIs of 6–7. This triad was also seen in the RAW117-P fraction R (Table I), but the apparent M_r for both the DNase-I-treated and -untreated P-derived fraction R were of slightly higher M_r (~ 12,000–13,500) and slightly more basic pI compared with the H10-derived R fraction triad (Tables I, II). Additionally, we noted DNP/RNP "streaks" in the apparent M_r range of ~ 21,000–32,000, pI ~ 7, that were noted only in the DNase-I-treated samples, an observation also applicable to the P-derived fraction R sample. Such streaks could arise by enzymatic perturbation of protein-DNA and/or protein-RNA in the DNP/RNP complexes. We also observed the characteristic presence of basic DNP/RNPs with a streak-like appearance in both the control and DNase-I-treated samples as well as several discrete acidic DNP/RNPs. The DNase-I-treated H10-derived fraction R (Fig. 3) differed from the P-derived fraction R in its discrete DNP/RNP of apparent M_r ~ 57,000 and pI ~ 5.2 and two basic DNP/RNPs of apparent M_r ~ 32,500 and ~ 33,000 and pI of ~ 8.6 and ~ 8.8, respectively, that were not present in the P-derived fraction R. In general, the H10-derived R control sample contained more basic DNP/

Fig. 3. SDS-PAGE of DNP/RNPs derived from RAW117-H10 fraction R. DNP/RNP complex fractions were analyzed by SDS-PAGE in the presence or absence of DNase-I treatment. **A:** 1-D SDS-PAGE analysis of DNP/RNP fractions. **Lane 1** and **lane 14** correspond to molecular weight markers; **lanes 2, 4, 6, 8, 10, and 12** are from untreated samples, whereas **lanes 3, 5, 7, 11, and 13** are from DNase-I-treated samples. Lanes 2 and 3, fraction S1; lanes 4 and 5, fraction M1; lanes 6 and 7, fraction S2; lanes 8 and 9, fraction M2; lanes 10 and 11, fraction 0.1K; lanes 12 and 13, fraction R. **B:** 2-D isofocusing/reducing SDS-PAGE analysis of DNP/RNP fraction R from H10 nuclei. **C:** 2-D isofocusing/reducing SDS-PAGE analysis of DNP/RNP fraction R from H10 nuclei after treatment with DNase-I.

TABLE I. Analysis of RAW117-P DNP/RNPs for Enzymatic Activities and Specific Genes

Complex fractions[a]	M_r ($\times 10^{-3}$)	pI	DNase-I treated	Assoc. genes	TCA-precipitable radioactivity (mean ± SEM) $\times 10^{-3}$		
					Control	+Actinomycin D[b]	+α-Amanitin[c]
M2	20	5.5	–	*p53*	392 ± 58.8	814 ± 21.6	143 ± 36.9
M2	25	5.2	+	ND[d]	216 ± 41.0	257 ± 25.3	149 ± 9.6
R	52	5.5	–	ND	25 ± 4.7	115.8 ± 1.5	49.5 ± 9.6
R	35.5	5.5	–	*abl*	27.6 ± 5.8	18.5 ± 2.0	17.7 ± 3.3
R	30	5.8	–	ND	1,300 ± 9.5	18.1 ± 3.2	42.9 ± 2.5
R	13.0	7.7	–	18s rDNA	29.0 ± 2.31	122.4 ± 0.4	7.5 ± 2.2
R	12.5	7.4	–	ND	33.5 ± 4.2	29.2 ± 5.4	27.5 ± 5.1
R	13.5	7.2	–	ND	11,497 ± 24.7	124.1 ± 1.6	27.1 ± 3.9
R	33.0	7.9	–	ND	30.9 ± 0.1	127.7 ± 1.9	26.2 ± 2.5
R	13.0	7.4	+	ND	17.5 ± 2.2	112.0 ± 2.7	21.9 ± 4.7
R	12.5	7.2	+	18s rDNA	19.2 ± 0.9	115.4 ± 1.1	29.8 ± 4.9
R	13.5	7.0	+	ND	30.6 ± 2.25	31.3 ± 2.50	25.7 ± 4.4
R	16.5	7.7	+	ND	51.6 ± 3.93	116.2 ± 3.6	34.4 ± 1.0
R	14.0	7.7	+	*abl, ras*	12.0 ± 0.6	7.03 ± 0.9	9.02 ± 1.9
R	21.0	8.8	+	ND	18.8 ± 4.21	11.7 ± 0.37	13.2 ± 0.6
R	32	5.2	+	ND	103 ± 8.45	64.2 ± 3.7	47.4 ± 12.5
R	29	5.6	+	*abl*	5.8 ± 0.4	8.61 ± 0.2	7.0 ± 0.1
R	24	5.2	+	ND	14.3 ± 2.1	98.54 ± 1.4	9.02 ± 1.9
R	31	7.7	+	ND	29.7 ± 4.6	320.2 ± 3.8	22.3 ± 6.3
R	31	8.3	+	ND	36.8 ± 2.5	44.07 ± 1.2	24.1 ± 4.5
R	26	7.2	+	ND	31.6 ± 0.4	222.1 ± 2.1	34.3 ± 3.9

[a]DNA/RNP complex fractions; see Materials and Methods.
[b]20 × (w/w) excess.
[c]200 × (w/w) excess.
[d]Not detectable.

RNPs than did the P-derived fraction R. The 0.1K fraction from *Msp*-I-digested H10 nuclei contained only a few DNP/RNPs (Tables I, II). We found only one faint DNP/RNP in the control complex preparation, but DNase-I treatment yielded five additional DNP/RNPs, suggesting a tight protein-DNA association in the precursor complex. Three of the DNP/RNPs of apparent M_r ~14,000–17,000 and pI ~8.0 appeared as streaks in the DNase-I-treated samples in both the H10- and P-derived R fractions. As mentioned above, we speculate that the streak-like pattern is probably due to DNA- or RNA-protein interactions in the precursor DNP/RNP complex that are perturbed by enzymatic treatment. However, more data will be needed to confirm this notion. In addition, the DNase-I-treated 0.1K fraction showed two low abundance acidic DNP/RNPs, corresponding in apparent size and pI to those found in fraction R from H10 nuclei. As we did not detect any initial positive hybridization signals in DNA purified from the precursor P-derived fractions 0.1K and M1, we excluded these fractions from our initial studies. The H10-derived fraction M1 was characterized by the presence of two high apparent M_r DNP/RNPs (~140,000, ~120,000) and pI ~5.8 in both the control and DNase-I-treated samples (Table II). In the DNase-I-treated sample, an array of low abundance DNP/RNPs was apparent. The isofocusing pattern corresponding to DNP/RNP complex fraction M2 was less complex, with only one DNP/RNP visible in the DNase-I-treated sample of apparent M_r ~30,000 and pI ~5.5. On the other hand, the P-derived fraction M2 contained DNP/RNPs in both the untreated and DNase-I-treated samples. The untreated P-derived fraction M2 contained one detectable DNP/RNP of apparent M_r ~20,000 and pI ~5.5, whereas the DNase-I-treated fraction contained an DNP/RNP of apparent M_r ~25,000 and pI ~5.2 (Table I).

Identification of Genes in Isolated DNP/RNPs

To identify specific DNA sequences in the DNP/RNPs isolated from RAW117-H10 and -P nuclei as described in Materials and Methods, we chose gene probes that gave positive hybridization signals for phenol-purified, ethanol-precipitated DNA derived from the appropriate

TABLE II. Analysis of RAW117-H10 DNP/RNPs for Enzymatic Activities and Specific Genes

Complex fractions[a]	M_r ($\times 10^{-3}$)	pI	DNase-I treated	Assoc. genes	TCA-precipitable radioactivity (mean ± SEM) $\times 10^{-3}$ Control	+Actinomycin D[b]	+α-Amanitin[c]
M1	140	5.8	–	*p53*[d,f]	20.1 ± 2.7	17.8 ± 2.9	15.7 ± 0.8
M1	120	5.8	–	*p53,abl*[f]	21.8 ± 0.3	17.8 ± 22	13.9 ± 3.5
M1	68	5.8	–	*p53, abl*	38.3 ± 6.3	22.3 ± 2.1	28.6 ± 5.1
M1	53	5.2	–	ND[e]	74.1 ± 7.6	48.9 ± 6.1	82.1 ± 9.6
M1	32	5.2	+	ND	129. ± 2.7	385 ± 11.0	154 ± 14.5
M1	140	5.8	+	*p53*[f]	48.4 ± 2.4	50.8 ± 0.9	58.0 ± 9.8
M1	120	5.8	+	ND	42.6 ± 6.6	55.4 ± 7.0	67.1 ± 0.4
M1	68	5.8	+	ND	60.3 ± 12.1	114 ± 28.4	90.5 ± 4.6
M1	53	5.2	–	18s rDNA	47.9 ± 3.29	46.6 ± 1.8	49.1 ± 4.9
M1	32	5.2	+	ND	41.4 ± 9.8	39.4 ± 5.2	44.0 ± 5.2
M1	31	5.5	+	ND	34.6 ± 0.8	39.0 ± 2.7	50.7 ± 3.6
M1	30	5.7	+	μ-Chain Ig	41.0 ± 6.1	32.4 ± 2.2	40.1 ± 8.2
M1	30	5.0	+	ND	47.6 ± 2.5	48.2 ± 0.6	61.7 ± 2.7
M1	24.5	5.1	+	ND	43.1 ± 2.5	20.1 ± 5.1	30.4 ± 6.7
M1	17	6.7	+	ND	30.3 ± 9.2	28.8 ± 5.1	80.2 ± 4.6
M1	16	7.0	+	ND	33.6 ± 3.3	42.1 ± 1.3	54.2 ± 12
M1	14.5	7.3	+	ND	26.8 ± 1.3	38.1 ± 9.2	34.8 ± 0.3
M1	24	5.1	+	ND	53.5 ± 3.3	33.2 ± 1.1	44.5 ± 4.1
0.1K	28	5.5	+	ND	99.3 ± 10.1	139 ± 25.8	117 ± 19.4
0.1K	50	5.2	+	ND	112 ± 24.1	95.3 ± 27.6	110 ± 25.4
0.1K	34	5.5	–	μ-Chain Ig	33.3 ± 5.4	40.0 ± 0.7	30.5 ± 0.8
0.1K	17	8.0	+	ND	106 ± 8.1	150 ± 2.7	129 ± 11.9
0.1K	16	8.0	+	ND	79.0 ± 6.1	93.3 ± 5.4	101 ± 13.5
0.1K	13	8.0	+	*p53*	34.3 ± 5.6	45.7 ± 3.8	10.8 ± 0.3
R	12	7.5	–	ND	132 ± 17.0	192 ± 3.5	141 ± 4.0
R	11.5	7.3	–	ND	168 ± 2.2	281 ± 54.9	183 ± 20.0
R	12.5	7.0	–	ND	248 ± 36	242 ± 26.5	233 ± 35.1
R	13.0	7.3	+	ND	361 ± 53.2	484 ± 63.6	360 ± 77.8
R	12.0	7.0	+	ND	240 ± 32.1	346 ± 15.6	261 ± 35.0
R	13.5	6.7	+	ND	286 ± 22.4	1,058 ± 123	244 ± 22.9
R	57	5.2	–	*p53*	1,540 ± 340	1,577 ± 135	1,742 ± 26.3
R	57	5.2	+	*p53, abl*[f]	635 ± 24.4	740 ± 9.4	1,480 ± 25.5
R	30	5.7	–	ND	860 ± 33.0	303 ± 20.0	1,099 ± 243
R	30	5.4	+	*p53, abl,* μ-Ig, *neu, ras*	504 ± 59.7	610 ± 22.7	501 ± 38.8
R	53	7.2	–	ND	129 ± 1.41	150 ± 32.4	71.8 ± 10.9
R	57	7.0	+	*abl*	103 ± 7.1	66.8 ± 22.4	36.6 ± 4.1
R	32	7.8	–	*p53*	60.8 ± 22.2	102 ± 18.5	47.7 ± 5.2
R	33	7.8	+	ND	248 ± 3.8	149 ± 11.3	30.3 ± 2.8
R	32.5	8.6	–	*abl*	17.2 ± 0.8	31.4 ± 0.8	85.3 ± 1.8
R	32.5	8.6	+	*abl*	85.5 ± 1.65	228 ± 0.1	14.5 ± 0.1
R	33	8.8	–	ND	321 ± 1.0	119 ± 0.7	14.4 ± 0.50
R	33	8.8	+	ND	89.1 ± 4.0	45.6 ± 3.2	61.3 ± 0.8
R	35	5.2	–	μ-Chain Ig, 18s rDNA	825 ± 9.7	704 ± 62.5	387 ± 79.3
R	17	8.0	+	ND	22.2 ± 2.2	43.7 ± 0.1	172 ± 6.2
R	16	8.0	+	ND	57.5 ± 5.9	33.4 ± 3.9	96.7 ± 22.9
R	14	8.0	+	ND	226 ± 12.3	654 ± 32.8	43.1 ± 0.06

[a]DNA/RNP complex fractions; see Materials and Methods.

[b]20× (w/w) excess.

[c]200× (w/w) excess.

[d]pg levels of β-casein were not detected, but back-hybridization with the in vitro synthesized RNA indicated the presence of β-casein.

[e]Not detectable.

[f]pg levels of c-H-*ras* and c-*neu* DNA were not detected, but back-hybridization with DNP/RNP reconstitutes of in vitro synthesized RNA indicated the presence of c-H-*ras* and/or c-*neu*.

complex fractions (data not shown). RAW117-P-derived DNP/RNP complexes fraction R DNA gave positive signals for c-H-*ras*, c-*neu*, 18s rDNA, and v- or c-*abl*. Since DNA from RAW117-H10 fractions R, 0.1K, M1, and M2 yielded positive hybridization signals for c- or v-*abl*, *p53*, 18s rDNA, and μ-chain immunoglobulin genes, we screened these fractions after further separation by 2-D isofocusing/SDS-PAGE. For the hybridization experiments, the same dot-blot was stripped and used for hybridization with the genes of interest to minimize error in DNA quantitation or fluctuation in the quantities of DNP/RNPs from different blots (Fig. 4). A representative dot-blot pattern for *p53* (Fig. 4B) and μ-chain immunoglobulin gene (Fig. 4C) is shown juxtaposed to the dot-blot format (Fig. 4A). Those DNP/RNPs shown to be hybridization-positive for pg detectable levels of the genes tested are summarized in Tables I and II. None of the DNP/RNPs tested positive for the presence of the control pSV2*neo* gene. Eight DNP/RNPs were positive for genes in the dot-blot hybridization studies that were *not* derived from precursor DNP/RNP complexes pretreated with DNase-I. The apparent M_r range for these eight DNP/RNPs was ~20,000–~120,000 and the pI range was ~5.2–8.6. Those DNP/RNPs that were derived from DNP/RNP complexes that were pretreated with DNase-I had a similar pI range but their apparent M_r range was even broader (~13,000–~140,000). *p53* DNA was detected in DNP/RNPs of apparent M_r ~20,000, pI ~5.5; apparent M_r ~13,000, pI ~8.0; two DNP/RNPs of identical apparent M_r and pI, one treated with DNase-I, the other untreated (apparent M_r ~57,000, pI ~5.2); apparent M_r ~30,000, pI ~5.4; apparent M_r ~57,000, pI ~7.0; apparent M_r ~32,500, pI ~8.6; apparent M_r ~120,000, pI ~5.8; and apparent M_r ~140,000, pI ~5.8. Immunoglobulin μ-chain gene was detected in DNP/RNPs of apparent M_r ~34,000, pI ~5.5; apparent M_r ~13,000, pI ~8.0; apparent M_r ~35,000, pI ~5.2; apparent M_r ~30,000, pI ~5.7. The gene for 18s rDNA was also detected in the latter DNP/RNP (data not shown). Since the gene for μ-chain immunoglobulin is next to the rDNA genes, we expected that some of the μ-chain immunoglobulin-containing DNP/RNPs would also contain rDNA genes [Arnheim et al., 1980]. A positive hybridization signal for c-*abl* was observed for seven DNP/RNPs, and c-H-*ras* was found only in DNP/RNPs of apparent M_r ~30,000, pI ~5.4 and

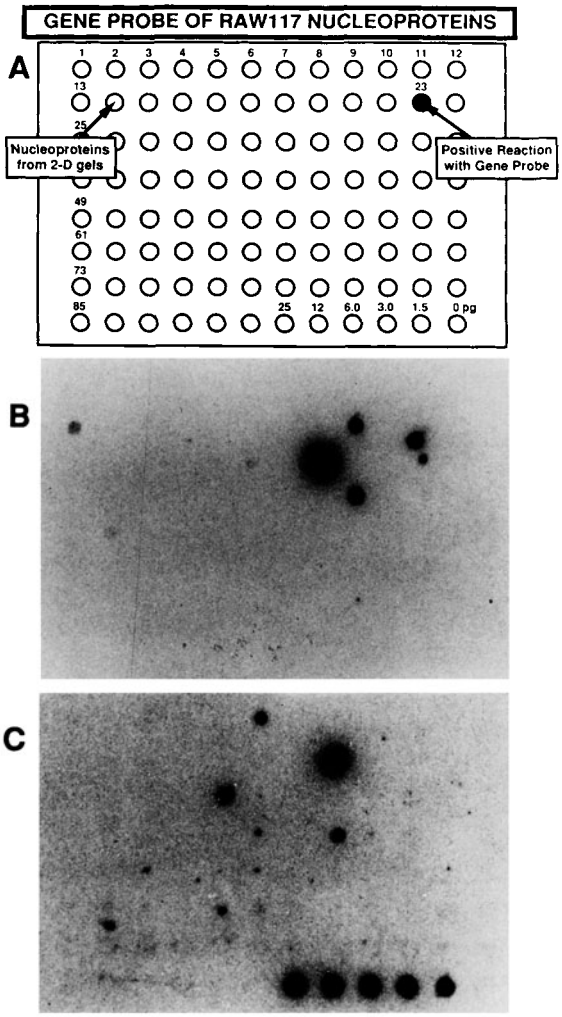


Fig. 4. Dot-blot hybridization for detection of specific genes in isolated DNP/RNPs. The arrangement of spotted DNP/RNPs and concentration controls are illustrated in **A.** Isofocusing/SDS-PAGE-purified DNP/RNPs were spotted onto Nytran and screened for the following genes: v-*abl*, *p53*, μ-chain immunoglobulin, c-H-*ras*, c-*neu*, and β-casein. The data for *p53* (**B**) and μ-chain immunoglobulin (**C**) genes are shown juxtaposed to the dot-blot format (A). A quantitative control corresponding to known dilutions of purified total RAW117 cellular DNA is present in the bottom lane (see A). DNP/RNPs containing genes of interest are summarized in Tables I and II.

apparent M_r ~33,000, pI ~7.8. Only one DNP/RNP of apparent M_r ~30,000, pI ~5.4 elicited a positive signal for c-*neu* in the H10-derived R fraction. Since we observed that the DNP/RNP of apparent M_r ~30,000, pI ~5.4 showed positive hybridization for several of the genes studied, we initially concluded that this DNP/RNP was a nonspecific DNA-binding protein. However, it did not bind to nonspecific DNA sequences or the gene probe for 18s rDNA (unpublished DNP/RNP observations). Since c-H-*ras*

and c-*neu* were observed only in the DNase-I-treated H10-derived R fraction sample, c-H-*ras* and c-*neu* may be tightly bound and relatively inaccessible before DNase-I treatment. On the other hand, *abl*, *p53*, and immunoglobulin μ-chain genes were present in both moderately to tightly bound, accessible DNase-I-sensitive DNA sequences as well as tightly bound inaccessible sequences. Interestingly, *p53* was enriched in particular DNP/RNPs compared to the total cellular DNA, since we were never able to get a positive hybridization signal in the latter at comparable DNA loads (Fig. 4B). Other genes (such as π-glutathione-S-transferase) analyzed in these studies yielded positive signals in the control cellular DNA, but only when enough DNA was loaded (data not shown).

Treatment with DNase-I affected gene detection in some of the isolated DNP/RNPs. For example, the *abl* gene was not found in detectable quantities in an DNP/RNP of apparent M_r ~57,000, pI ~5.2 (H10-R), and was detected only after the complex was treated with DNase-I. This suggests that the *abl* DNA sequences in this DNP/RNP could be in a highly folded structure that is inaccessible to probe unless perturbed with DNase-I. We cannot completely rule out, however, that pretreatment of the precursor complexes may have promoted the nonspecific interaction of the *abl* gene probe to other less abundant DNP/RNPs in the same precursor DNP/RNP complex.

In Vitro RNA Pol and Primase in Isolated DNP/RNPs

We screened over 450 DNP/RNPs for in vitro enzymatic activities, but only DNP/RNPs that were positive for particular genes are included in this report (Tables I, II). We excluded the DNP/RNPs that contained modest in vitro RNA pol and RNA primase activities, under conditions used in these studies, because of the possibility that these activities were due to in vitro nuclease and/or other activities. Although indicative of the presence of RNA pol-like activity, high levels of incorporation of [α^{32}P]GTP by particular DNP/RNPs were not necessarily proof of specific in vitro transcription competency. Some of the DNP/RNPs showed high RNA pol-like activities in vitro. Eight DNP/RNPs whose RNA pol-like activity was shown to be especially sensitive to 200× (w/w) excess α-amanitin, were designated to contain RNA pol II- and III-like activities. Thirteen DNP/RNPs were determined to be particularly α-amanitin-resistant, suggesting the presence of an RNA pol I-like activity. DNP/RNPs of apparent M_r ~20,000, pI ~5.5 (P-M2), apparent M_r ~53,000, pI ~7.2 (H10-R), and apparent M_r ~33,000, pI ~8.8 (H10-R) were determined to contain both RNA pol I- and II/III-like activities, because ~30–40% of the activity persisted in the presence of excess α-amanitin. DNase-I treatment of the DNP/RNP of apparent M_r ~33,000, pI ~8.8 (H10-R) promoted the detection of RNA pol I-like activity that was not detected in the untreated sample. An analogous observation was made with the DNP/RNP of apparent M_r ~32,500, pI ~8.6 (H10-R) and RNA pol II-like activity. RNA primase-like activity was detected in nine DNP/RNPs. Under the conditions employed in these studies, treatment with DNase-I did not abolish RNA primase-like activity, as was the case for the DNP/RNP of apparent M_r ~33,000, pI ~7.8 (H10-R) (Table II).

We were at first surprised to find the RNA pol and primase activities in DNP/RNPs of relatively low molecular weight, because RNA pol activity has been attributed to higher M_r weight components [Buhler et al., 1986]. Particularly interesting was the observation of α-amanitin-resistant activity noted for DNP/RNPs with apparent M_rs much lower than the principal characterized RNA pol I component of 200 kDa [Buhler et al., 1986]. Since migration in a SDS-PAGE gel is not an absolute measurement of size, these DNP/RNPs may exhibit anomalously low electrophoretic mobilities and/or are a different but related novel subset of highly compacted RNA pol/primase components than those previously studied.

Dot-blot hybridization experiments (Fig. 4) indicated that DNP/RNPs of apparent M_r ~34,000, pI ~5.5 (H10-0.1K), apparent M_r ~120,000, pI ~5.8 (H10-M1), and apparent M_r ~30,000, pI ~5.7 (H10-M1) contained detectable quantities of μ-chain immunoglobulin gene, whereas one DNP/RNP of apparent M_r ~120,000, pI ~5.8 (H10-M1) also contained *p53* and *abl* genes, respectively (Table II). Arnheim et al. [1980] demonstrated that the μ-chain immunoglobulin gene is proximal to the ribosomal genes, so the observation that the μ-chain gene is detected in an DNP/RNP that shows RNA pol I-like activity and the presence of the 18s rDNA gene is not surprising. However, other DNP/RNPs that contained the 18s rDNA gene did not concomitantly possess RNA pol I-like

activity. RNA pol I- and II-like activities were detected with an DNP/RNP of apparent M_r ~20,000, pI ~5.5 (P-M2) shown to contain *p53,* whereas RNA pol II-like activity was detected with an DNP/RNP of apparent M_r ~32,000, pI ~5.2 (P-R) that contained the *abl* gene. RNA pol II-like activity was also detected weakly in an DNP/RNP of apparent M_r ~57,000, pI ~7.0. We were not able to detect strong RNA pol I-like activity in certain individual DNP/RNPs with detectable 18s rDNA gene, suggesting that combinations of the DNP/RNPs may be required to reconstitute the complete activity. Interestingly, we did detect RNA pol I-like activity in an DNP/RNP of apparent M_r ~30,000, pI ~5.4 (H10-R) that was shown to contain *p53, abl,* and μ-chain gene but not β-casein gene. We initially concluded that this DNP/RNP may have bound the probes nonspecifically, because it also elicited a signal for the human gene *Bcl-2* by dot-blot hybridization (unpublished observations). Purified total cellular DNA and 18s rDNA, though, did not hybridize to this DNP/RNP.

We were able to detect significant RNA primase-like activities with some of the gene-containing DNP/RNPs and demonstrated that these were sensitive to actinomycin D. These DNP/RNPs were a *p53*-containing DNP/RNP of apparent M_r ~20,000, pI ~5.5 (P-M2); 18s rDNA-containing DNP/RNPs of apparent M_r ~33,000, pI ~8.8 (H10-R) and apparent M_r ~12,500, pI ~7.2 (P-R), respectively; and an *abl*-containing DNP/RNP of apparent M_r ~32,000, pI ~5.2 (P-R) (Tables I, II). As was the case with RNA pol I- and II-like activities, no simple correlation was found between the presence of a particular gene and coincident presence of both the RNA primase- and pol-like activities. Thus we did not conclude that the presence of the appropriate RNA pol- and primase-like activities in conjunction with the presence of a gene necessarily rendered an individual DNP/RNP transcriptionally competent.

DNP/RNP Reconstitution and Back-Hybridization of RNA Pol Products

The purpose of the back-hybridization studies was to determine if particular DNP/RNP complex reconstitutes or individual DNP/RNPs were capable of producing a RNA product in vitro that would hybridize back to specific genes. Several hundred individual DNP/RNPs and combinations of different DNP/RNPs were examined, but we found that only a few individual DNP/RNPs or combinations of DNP/RNPs produced RNA pol products that hybridized back to the genes we studied. Active genes investigated in the RAW117 system were *abl, p53,* and μ-chain gene, and genes known to be inactive or expressed at low levels in this system were β-casein, c-H-*ras,* and c-*neu*. The antibiotic resistance gene *neo* served as a negative control. Extracellulose chromatography-purified DNP/RNP RNA products (Fig. 5A) served as back-hybridization probes and were hybridized to defined amounts of specific cDNAs spotted on Nytran as described in Materials and Methods. The inclusion of actinomycin D in the in vitro RNA synthesis reactions inhibited in the majority product formation (Fig. 5B), suggesting the presence of RNA primase-like initiation capability.

Only specific combinations of DNP/RNPs or certain individual DNP/RNPs resulted in the production of RNA products capable of back-hybridization to the cDNAs, while other combinations yielded weakly positive signals with the immobilized genes (Fig. 6). Using DNP/RNPs of apparent M_r ~34,000, pI ~5.5, M_r ~35,000, pI ~5.2, and M_r ~29,000, pI ~5.6, we found synthesis of both *p53* and μ-chain gene RNAs (Fig. 6, lane 1), whereas these individual DNP/RNPs assayed alone did not produce sufficient RNA product to yield a positive result in the back-hybridization assay. Other combinations of the DNP/RNP of M_r ~34,000, pI ~5.5, e.g., with DNP/RNPs of M_r ~35,000, pI ~5.2, and M_r ~19,000, pI ~8.0, did not yield positive back-hybridization (Fig. 6, lane 3). From the dot-blot hybridization, we observed that the DNP/RNP of apparent M_r ~29,000, pI ~5.6 was positive for *abl,* and DNP/RNPs of apparent M_r ~34,000, pI ~5.5, and M_r ~35,000, pI ~5.2, were positive for μ-chain gene. Because DNA sequences containing the *abl* gene were part of the reconstituted DNP/RNP complex, we conclude that under these in vitro conditions specific DNP/RNPs were regulating the synthesis of *p53* and μ-chain mRNA. On the other hand, it could be that *abl* mRNA as well as *abl* DNA sequence may be involved in the in vitro regulation of β-casein RNA. For example, an isolated DNP/RNP of apparent M_r ~140,000, pI ~5.8 was individually capable of synthesizing RNA (Fig. 5A, lane 4) that hybridized back to the β-casein gene (Fig. 6, lane 4), which is not normally expressed in the RAW117 system. This DNP/RNP simultaneously synthesized a small amount of μ-chain and *abl* RNA, as indicated by weak signals with

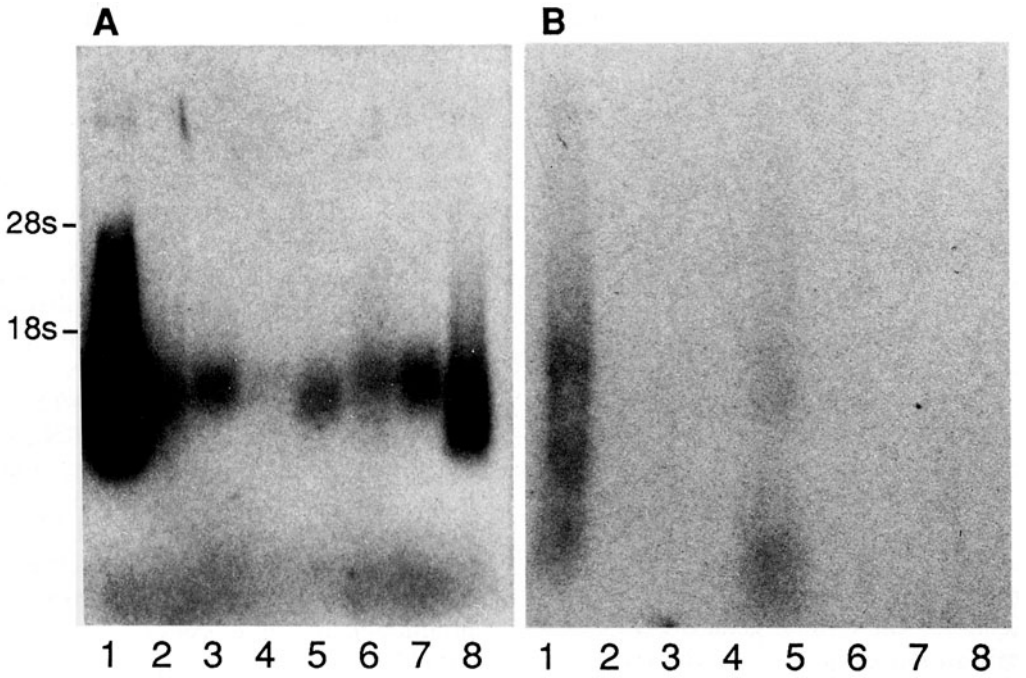


Fig. 5. Glyoxyl gel electrophoresis of purified DNP/RNP RNA synthesis products which were used as back-hybridization probes. **A:** In vitro RNA products generated by DNP/RNPs in the absence of actinomycin D. **B:** Comparable reactions conducted in the presence of actinomycin D. Some elongation-like activity persists for in vitro products formed by the reactions, as shown in lanes 1 and 5. **Lane 1,** RNA products from a mixture of DNP/RNPs of apparent M_r ~30,000, pI ~5.4, M_r ~21,000, pI ~8.8, and M_r ~31,000, pI ~7.6; **lane 2,** RNA products from DNP/RNPs of apparent M_r ~34,000, pI ~5.5, M_r ~35,000, pI ~5.2, and M_r ~57,000, pI ~5.2; **lane 3,** RNA products from DNP/RNPs of apparent M_r ~34,000, pI ~5.5, M_r ~35,000, pI ~5.2, and M_r ~29,000, pI ~5.2; **lane 4,** RNA products from an individual DNP/RNP of apparent M_r ~140,000, pI ~5.8; **lane 5,** RNA products from an individual DNP/RNP of apparent M_r ~21,000, pI ~8.8; **lane 6,** RNA products from an individual DNP/RNP of apparent M_r ~31,000, pI ~7.6; **lane 7,** products of DNP/RNP reconstitutes of apparent M_r ~140,000, pI ~5.8, apparent M_r ~120,000, pI ~5.8, and apparent M_r ~37,000, pI ~5.2; **lane 8,** RNA products of DNP/RNP of apparent M_r ~140,000, pI ~5.8 (DNase-I treated).

immobilized v-*abl* and μ-chain cDNAs (Fig. 6, lane 4). We interpret the β-casein result as follows. The DNP/RNP of apparent M_r ~140,000, pI ~5.8 was removed from its normal regulatory controls, some of which may be provided by macromolecular associations with other DNP/RNPs. Therefore, the ability of the DNP/RNP to synthesize β-casein mRNA was apparently promoted by removing other adjoining DNP/RNPs. Interestingly, we found that other DNP/RNP combinations modified the synthesis of specific RNAs. For example, reconstituting DNP/RNPs of M_r ~140,000, pI ~5.8 (DNase-I-treated) with DNP/RNPs of M_r ~120,000, pI ~5.8 and M_r ~57,000, pI ~5.2 promoted the synthesis of c-H-*ras* but suppressed the synthesis of c-*neu* RNA (Fig. 6, lane 7).

DISCUSSION

We report here on the further characterization of DNP/RNP complexes (repliscriptons) derived from direct digestion of nuclei with *Msp*-I [Rosenberg-Nicolson and Nicolson, 1992]. Some of the constituent DNP/RNPs of RAW117 nuclei were shown to contain tightly bound gene template DNA sequences for *abl, p53,* immunoglobulin μ-chain, c-H-*ras,* 18s rDNA, and c-*neu* genes. A precedent for this type of nucleoprotein has been shown by studies with the zinc-finger protein TFIIIA that contains covalently bound DNA and/or RNA and can bind soluble factors [Peck et al., 1987; Gottesfeld et al., 1987; Blanco and Gottesfeld, 1988; Engelke and Gottesfeld, 1991]. We found that DNP/RNPs containing tightly bound template appeared to be primarily associated with precursor DNP/RNP complexes hypothesized to be from more topographically buried chromatin regions (DNP/RNP complex fractions R, M1, and M2), although β-casein DNA was detected in the S1 fraction (data not shown). Back-hybridization studies using purified in vitro RNA pol products from a variety of

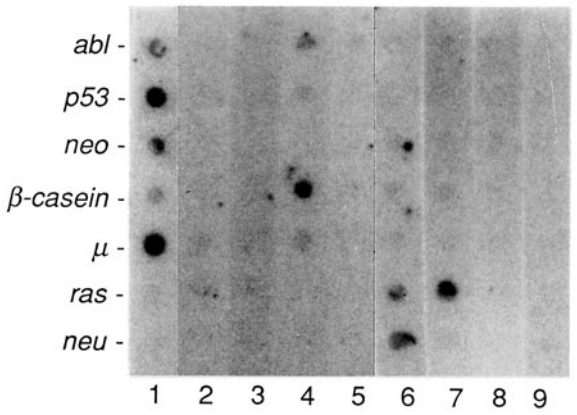


Fig. 6. Back-hybridization of RNA produced by individual DNP/RNPs or DNP/RNP reconstitutes to specific gene cDNAs immobilized on Nytran. Individual DNP/RNPs and reconstituted DNP/RNP complexes were allowed to synthesize RNA, and the products purified and back-hybridized to specific gene cDNAs as described in Materials and Methods. **Lane 1,** RNA from a DNP/RNP reconstitute consisting of DNP/RNPs of apparent M_r ~34,000, pI ~5.5, M_r ~35,000, pI ~5.2, and M_r ~29,000, pI ~5.2 was back-hybridized to various cDNAs; **lane 2,** RNA from a DNP/RNP reconstitute consisting of DNP/RNPs of apparent M_r ~34,000, pI ~5.5, M_r ~31,000, pI ~5.2, and M_r ~68,000, pI ~5.8 was back-hybridized to various cDNAs; **lane 3,** RNA from a DNP/RNP reconstitute consisting of DNP/RNPs of apparent M_r ~34,000, pI ~5.5, M_r ~35,000, pI ~5.2, and M_r ~19,000, pI ~8.0 was back-hybridized to various cDNAs; **lane 4,** RNA from a DNP/RNP of M_r ~140,000, pI ~5.8 was back-hybridized to various cDNAs; **lane 5,** RNA from a DNP/RNP of M_r ~31,000, pI ~7.7 was back-hybridized to various cDNAs; **lane 6,** the DNP/RNP used as in lane 4, but in combination with DNP/RNPs of apparent M_r ~57,000, pI ~5.2, and M_r ~120,000, pI ~5.8; **lane 7,** RNA from a DNP/RNP of M_r ~140,000, pI ~5.8 (DNase-I treated) with DNP/RNPs of M_r ~57,000, pI 5.2 and M_r ~120,000, pI 5.8 were back-hybridized to various cDNAs; **lane 8,** RNA from a DNP/RNP reconstitute consisting of DNP/RNPs of M_r ~140,000, pI ~5.8 and M_r ~30,000, pI ~5.4 were hybridized to various cDNAs; **lane 9,** RNA from a DNP/RNP reconstitute consisting of DNP/RNPs of M_r ~34,000, pI ~5.5, M_r ~35,000, pI ~5.2 and M_r ~16,000, pI ~8.0. Note that the substitution of a DNP/RNP in the reconstitute inhibited the synthesis of specific detectable RNA product.

SDS-PAGE-purified DNP/RNP complex reconstitutes and individual DNP/RNPs suggested that some DNP/RNPs were still capable of in vitro transcription initiation. Some of the DNP/RNPs, we speculate, then retain the correct mRNA start and stop sequences even after the isolation and purification. Since the DNP/RNPs in these studies possessed smaller apparent M_r than the RNA pol components of the well-characterized RNA pol I and II [Buhler et al., 1986], we propose that these DNP/RNPs migrate anomalously in the SDS-PAGE system and/or are a novel subset of the larger RNA pols which have been studied intensively. Relatively small RNA pols with analogous in vitro function to the larger, well-characterized RNA pols have been observed in prokaryotes [Ahn et al., 1990]. The eukaryotic DNP/RNPs studied here may contain analogous lower M_r RNA pols and/or RNA pols highly compacted by nucleic acid. Additionally, our observations suggest that the DNP/RNPs contained tightly bound RNA as well as DNA template.

Studies conducted by Chernokhvostov et al. [1989] previously demonstrated that eukaryotic chromatin contains a specific type of tightly bound RNA that was later speculated to be critical to the maintenance of an active chromatin radial loop structure. Since the DNP/RNPs studied here survived treatment with boiling SDS and urea, we speculate that these DNP/RNPs may be related to previously described chromatin substructures, such as those reported by Chernokhvostov et al. [1989]. Our unpublished observations indicate that the isofocusing/SDS-PAGE-purified DNP/RNPs (after removal of SDS) form distinctive spherical particle structures. We also found that the DNP/RNP RNA products used as back-hybridization probes for specific genes were sensitive to actinomycin D, suggesting that the DNP/RNPs possess in vitro transcription initiation capabilities. The back-hybridization technique using purified RNA pol products as probes to various cDNAs of interest enabled us to detect a β-casein mRNA product that is not normally expressed in RAW117 cells. Thus we speculate that our isolation and reconstitution procedures promoted the synthesis of a β-casein RNA pol product by perturbing the macromolecular associations of DNP/RNP proteins, RNAs, and DNAs. That DNP/RNP macromolecular conformational changes can occur and are directly involved in in vitro regulatory processes is possible, but additional research will be needed to confirm this notion. Our investigation was not designed to determine the in vitro regulatory controls involved in mRNA synthesis. We can only speculate that the DNP/RNPs with tightly bound DNA templates capable of being read by DNP/RNP pols contain a subset of the transcription and/or RNA pol factors that have been studied in a variety of in vitro systems, possibly as trans-acting factors. We found that one DNP/RNP reconstitute was capable of synthesizing an enormous mRNA (>60s in size), suggesting that some DNP/RNPs may function in a capac-

ity prior to mRNA processing, whereas other DNP/RNPs may be capable of in vitro translation and could be directly involved in RNA processing (unpublished observations).

The most important observations we have made regarding the 450 DNP/RNPs that we have isolated and screened can be summarized as follows: 1) the in vitro RNA pol/primase-like functions are retained after isolation of DNP/RNPs from isofocusing/SDS-PAGE gels and SDS removal; 2) the retained functions are complex and modulated by DNP/RNP interactions, perhaps indicative of another level of gene regulation, and some of the DNP/RNPs or mixtures of DNP/RNPs with tightly bound templates are capable of successful in vitro synthesis of RNAs that can hybridize with specific gene cDNAs; 3) the in vitro reconstitution of specific isolated DNP/RNPs demonstrated the synthesis of a normally "silent" gene (β-casein in RAW117 cells) that could be again "silenced" by combinations of specific DNP/RNPs; and 4) DNP/RNPs containing tightly bound template DNA were detected primarily in the R complex fraction from nuclei of the highly metastatic RAW117-H10 cell line.

Whether any of the in vitro observations made with the isolated DNP/RNPs of the repliscriptons are applicable to the in vivo state remains to be determined. Because the individual DNP/RNP isofocusing/SDS-PAGE patterns are relatively simple (either corresponding to a definitive spot or streak) compared to patterns often observed in whole cell-free extracts, and the in vitro functions can be directly assignable to an individual spot or streak of interest, future DNP/RNP analyses and studies involving the DNP/RNPs and their relationships to other functions are now possible and may eventually yield new insights into the silencing as well as expression of specific genes.

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