An agarose–acrylamide composite native gel system suitable for separating ultra-large protein complexes

Man-Hee Suh *, Ping Ye, Ajit B. Datta, Mincheng Zhang, Jianhua Fu *

Department of Molecular Biology and Genetics, Biotechnology Building, Cornell University, Ithaca, NY 14853, USA

Received 12 April 2005
Available online 8 June 2005

Abstract

An agarose–acrylamide composite native gel (CNG) system has been developed for separating protein complexes of ultra-large molecular sizes (over 500 kDa) and for analyzing protein–protein interactions in their native states. Various native gel conditions were explored and techniques were improved to facilitate the formation and performance of the CNG system. We demonstrate here that the CNG technique is capable of resolving a complex of RNA polymerase II and an associated factor from the free components, which had not been previously achieved with other methods. Furthermore, this CNG electrophoresis can be conveniently coupled to second-dimension sodium dodecyl sulfate–polyacrylamide gel electrophoresis for identification of protein components within discrete complexes separated during the CNG run. The CNG technique is particularly suitable for capturing dynamic protein–protein interactions as exemplified here by the formation and demonstration of RNA polymerase II–Fcp1 complex.

Keywords: Nondenaturing gel; Native gel; Composite native gel; Hybrid gel; RNA polymerase; Transcription factor; Protein complex

Polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions is one of the most commonly used methods for analyzing biological macromolecules such as nucleic acids, proteins, and their complexes [1]. In such native electrophoresis, separation of biomolecules is fulfilled on the basis of both their hydrodynamic (shape, size) and their electrostatic (net electrostatic charge) properties under the run condition, as opposed to separations based solely on hydrodynamic characteristics in gel-filtration (permeation) or size-exclusion chromatography (SEC) and on molecular weights in analytical ultracentrifugation (AUC). While SEC is suitable for determining macromolecular sizes and nondenaturing PAGE is not, nondenaturing PAGE has the advantage of stabilizing intermolecular interactions in the gel matrix. Native gel electrophoresis is therefore an important method for assessing formation and behaviors of macromolecular complexes, particularly when it is applied in combination with SEC and/or AUC. However, technical complications occur when ultra-large macromolecular species or assemblies need to be resolved in nondenaturing PAGE. In these cases, it is necessary to reduce acrylamide percentage dramatically to form meshwork loose enough to allow differential migration of individual biomolecules or their assemblies over distances adequate to permit analysis [2,3]. Though the lowest percentage of acrylamide that will allow gelation is around 3% (below which it remains fluid-like), in reality, gels made with less than 4% acrylamide are extremely difficult to work with. Nevertheless, the non-denaturing PAGE technique has proven most effective in the characterization of protein–DNA and protein–RNA

 Abbreviations used: CNG, composite native gel; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Pol II, RNA polymerase II; RNAP, E. coli RNA polymerase; SEC, size-exclusion chromatography; AUC, analytical ultracentrifugation; CTD, C-terminal repeated domain; DTT, dithiothreitol; BN, blue native; TEMED, N,N,N,N'-Tetramethylethylenediamine.
interactions, clearly due to the favorable charge characteristics, i.e., dominantly negative, of the oligomeric nucleic acids. In such studies, binding activity of a potential DNA- or RNA-recognizing protein is monitored as a change to the electrophoretic mobility of the nucleic acid labeled either isotopically or fluorescently [4]. However, if the binding protein has unusually large molecular size (say over 200 kDa), this technique suffers from practical constraints, as it becomes very difficult to produce useful migration for the protein–nucleic acid complex in polyacrylamide gels (typically ≥4%). This technical difficulty is well exemplified in the biochemico–structural work on the large-sized eukaryotic RNA polymerase II (Pol II; 500kDa), where agarose had to be used in place of polyacrylamide to identify the Pol II–DNA binary and Pol II–DNA–RNA ternary complexes [5]. This native gel technique using agarose proved to be a critical step in the series of studies that culminated in the resolution of a crystallographic structure of the transcription ternary complex [6].

Although native agarose gel electrophoresis worked sufficiently well for the study of Pol II ternary complex, its application to other large-sized protein assemblies devoid of nucleic acids puts forward additional practical problems including severe band diffusion due to longer running time (required in the absence of negative charges from nucleic acids) apart from the fact that these gels can be run only in horizontal settings, making it extremely difficult to couple with a second-dimension SDS–PAGE.

An obvious solution to the problems associated with either polyacrylamide or agarose gel matrix is to combine the useful properties from both of them. With such a thought, Uriel [7] first described a hybrid gel composed of both acrylamide and agarose, but gels with less than 3% of acrylamide could not be achieved in that study. Later, Peacock and Dingman [8] formulated a composite gel of 0.5% agarose and 2% acrylamide to separate large RNAs. More recently, Tatsume and Hattori [9] reported an agarose–acrylamide hybrid gel to facilitate SDS–denaturing electrophoresis of giant myofibrillar protein. Little has been done to further develop the composite gel technique due to rather complicated and delicate procedures involved in the handling.

In the course of characterizing yeast Pol II interactions with a number of associated factors [10,11], we encountered the need to effectively separate large-sized multiprotein assemblies formed between them. We therefore adopted the composite gel technique as a high-resolution method to separate large protein complexes and to study protein–protein interactions in their native states. We have made improvements with regard to sample well formation (using a triple-comb technique) and coupling to a subsequent denaturing SDS–PAGE for identifying the components of individual complexes separated during the native run. Using this composite native gel (CNG) protocol, we have been able to separate large complexes formed between Pol II and several regulatory proteins as demonstrated here with Fcp1, a phosphatase specific for the C-terminal repeated domain (CTD) of Pol II. These results suggest that the CNG protocol developed here should find successful applications in studying other multiprotein complexes also.

Materials and methods

Materials

Acrylamide stock solution (30% acrylamide, 37:1 cross-linking ratio) was purchased from National Diagnostics (GA, USA). TEMED was obtained from EM Biosciences. SeaKem Gold agarose (Cambrex) was used unless mentioned otherwise. All other chemical reagents were purchased from Sigma (MO, USA). Protean II xi gel combs (20-well with 0.5 mm thickness) were purchased from Bio-Rad. The High-Molecular-Weight Calibration Kit for native electrophoresis containing thyroglobulin, ferritin, catalase, and other proteins (Amersham Biosciences) was used as an internal mobility control. Prestained SDS-PAGE standards such as Broad Range (Bio-Rad) and Bench Mark Protein Ladder (Amersham Biosciences) were used as size markers for the second-dimension SDS–PAGE.

Preparation of protein samples

The 12-subunit RNA Pol II was purified from a protease-deficient Schizosaccharomyces pombe (S.p.) strain, CB010, using a TAP tag [12] fused to the C terminus of Rpb4 subunit (4TAP) as described previously [13]. Wild type and mutant (D172N) Schizosaccharomyces pombe (S.p.) Fcp1 proteins were expressed in Escherichia coli and purified as described [14] with an additional step of cation-exchange chromatography using a UNO-S column (Bio-Rad). Recombinant S.c. CTD fused to GST was also expressed in E. coli (using an expression plasmid kindly provided by Dr. R. Kornberg, Stanford University) and purified using glutathione Sepharose-4B (Amersham Biosciences). Phosphorylated 12-subunit Pol II (Pol IIo) was prepared by in vitro kinase reaction with p42 MAP kinase 2 (Erk2) from NewEngland Biolabs using the manufacturer’s recommended protocol with modifications. Briefly, 40 μg of purified 12-subunit Pol II was incubated with roughly 2000 units of the kinase in the presence of 100μM ATP (Amersham Biosciences) at 30°C for 4 h in a total volume of 250μl. Complete phosphorylation of Pol II was verified by (1) a discrete and maximum gel-mobility shift of the Rpb1 band in SDS–PAGE (6%) and (2) Western blotting of the phosphorylated Rpb1 using a phospho-CTD-specific antibody H14 (specific for the CTD phosphorylated at its serine-5 positions). The Pol IIo sample was repurified over calmodulin affinity resin (which binds
to a calmodulin binding peptide fused to the Pol II Rpb4 subunit) to remove free ATP and the kinase. E. coli holo and core RNA polymerases and the prokaryotic transcription antiterminator αQ protein were kindly provided by Dr. J. Roberts of Cornell University. All proteins were transferred to appropriate buffers and concentrated using centrifugal ultrafiltration devices (Nanosep, PALL or Centrifree, Millipore).

**Agarose–acrylamide composite gel preparation**

The composition of the CNG mix is given in Table 1. The gels were polymerized essentially following Peacock and Dingman [8] and Tatsumi and Hattori [9] with modifications as described below. A solution of 1% agarose (0.3 g/30 ml) was made in a 125-ml Erlenmeyer flask with heating in a microwave oven for 1 min and swirling every 10–20 s to promote mixing and melting of the agarose. The flask was then capped and maintained at 45°C using a water bath to prevent gelation. The acrylamide solution was then prepared in a separate Erlenmeyer flask by adding requisite amounts (see Table 1) of deionized water, 30% acrylamide, 20× buffer, 30% glycerol, 1 M DTT, and 10% ammonium persulfate in this order. Optional metal ion was added to this solution when it is required for stabilizing the individual protein components or protein–protein interactions in the complex. The mixture was degassed for 1 min, capped, and then transferred to the 45°C water bath for about 20 min to reach thermal equilibration. After incubation, the acrylamide solution was poured into the flask containing the molten agarose, mixed by gentle swirling, and incubated at 45°C for 1 min. After adding 60 µl of TEMED, the gel mix was rapidly poured into an empty slab-gel assembly (200 × 140 × 1.5 mm), the slot-former was inserted, and the assembly was immediately transferred to 4°C for 10 min for the agarose to solidify, while the leftover gel mixture was maintained again in the 45°C bath. The cooled slab assembly was then taken out to room temperature, and the leftover gel mixture was used to fill the bottom gap after removing the bottom spacer. This eliminated the possibility of trapping air bubbles at the bottom of the gel. The slab assembly was then kept at room temperature for 1 h to allow full polymerization of the acrylamide. The prior gelation of the agarose by cooling at 4°C provided a vital mechanic support, since the polymerized acrylamide alone would still be fluid-like at these lower concentrations as reported [8,9].

**Formation of sample wells using triple comb**

Due to fragility of the composite gel and its gluey nature, it was necessary to use a triple-comb construct to facilitate the formation of sample wells. As shown in Fig. 1A, three Teflon combs each of 0.5 mm thickness were stacked with the top of the middle piece protruding. After the polymerization of the gel, the middle comb was removed and this removal facilitated the subsequent removal of the other two combs.

**Native electrophoresis**

The electrophoresis was carried out at 4°C using 1× gel buffer (containing the specified metal ions as and where mentioned). Usually the gel was precooled for 1 h at 4°C and prerun for 30 min at 150 V. While analyzing protein–protein interactions, protein mixtures (in 15 µl containing at least 10% glycerol) were incubated at a suitable temperature for 30 min and loaded into cleaned sample wells. An aliquot of nondenaturing gel loading buffer (15 µl) containing bromophenol blue dye was loaded in an empty lane and used as run indicator; bromophenol blue migration coincided with catalase from the native electrophoresis marker kit. The electric field (150 V) was applied until the dye reached the bottom of the gel. At the end of a run, the gel was gently pushed off the glass plate into a container containing Bradford reagent and stained for 30–60 min with slow rocking. The destaining was achieved with deionized water until the reddish background completely disappeared, which usually took a few hours. Alternatively, the gel was destained overnight in a large volume of deionized water. A properly destained composite gel showed virtually no background. Complete destaining was important because gels with incomplete destaining developed a dark brown background after drying with gel-drying films (Promega). Stained gels were routinely recorded by scanning with either regular optical scanners (e.g., HP ScanJet 4400c) or a Storm-860 laser scanning system (Amersham Biosciences).

**Second-dimension SDS–PAGE**

For carrying out second-dimension SDS–PAGE, the description by Schägger et al. [15,16] was followed with some modifications. Before the first-dimension gel was poured, extra spacers were inserted parallel to the side spacers with gaps of about 0.5–1.0 cm from each other (Fig. 1B). In these cases, it was particularly important to prewarm the empty slab assembly to ~40°C before

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composite native gel formulation (optimized conditions)</td>
</tr>
<tr>
<td>Reagent</td>
</tr>
<tr>
<td>1% Agarose</td>
</tr>
<tr>
<td>30% Acrylamide (37.5:1)</td>
</tr>
<tr>
<td>20× Gel buffer*</td>
</tr>
<tr>
<td>30% Glycerol</td>
</tr>
<tr>
<td>1 M DTT</td>
</tr>
<tr>
<td>10% Ammonium persulfate</td>
</tr>
<tr>
<td>1 M MgCl$_2$ (optional)</td>
</tr>
<tr>
<td>10 mM ZnCl$_2$ (optional)</td>
</tr>
<tr>
<td>TEMED</td>
</tr>
<tr>
<td>dd H$_2$O</td>
</tr>
<tr>
<td>Total volume</td>
</tr>
</tbody>
</table>

* Tris–borate, 0.45 M, pH 8.0 (adjusted with HCl).
pouring the gel material. Omission of this prewarming step resulted in premature solidification of the CNG mix due to accelerated cooling by the additional spacers. To avoid air bubbles trapped in the middle of the lane which would interfere with flow of the gel mix, the gel apparatus was slanted, and the mix was added slowly using a 1-ml micropipette. Alternatively, the extra spacers could be inserted without touching the bottom spacer (a gap of ~1 cm) and the composite solution was poured into the larger chamber, which automatically filled the strip. It is important to warm the empty assembly to ~40 °C prior to pouring the CNG mix to prevent premature solidification due to extra cooling effects from the additional spacers. (C) Setup for second-dimension SDS–PAGE. The SDS polyacrylamide gel is formed in the regular manner. Before the stacking gel solidifies, the gel strip from the CNG run is inserted on top of the stacking gel. After its polymerization, the stacking gel above the gel strip is removed using a pipette tip. Then, 6× SDS sample dye is loaded over the gel strip. During the initial stage of electrophoresis, SDS molecules from the SDS sample buffer denature the protein inside the CNG strip, eliminating the need to presoak the strip for denaturation.

Results

A triple-comb technique for forming sample wells in agarose–acrylamide CNG

Even though agarose was added to strengthen the acrylamide gel, we found that ordinary single-pieced
combs were unsuitable for forming sample wells, due to the fragility and the gluey nature of the composite gel. Usually, walls of the wells remained stuck on the comb, making it difficult to remove the comb without breaking the walls. Tatsumi and Hattori [9] solved this problem by introducing air into the sample wells with thin needles inserted between the walls and the comb. This technique worked for gels containing SDS, which provided slippery traits to the gel. But it was impossible to apply this technique to CNG due to the sticky behavior of the gel material. To circumvent this problem, we constructed a stacked triple comb (depicted in Fig. 1A). Following gel polymerization, the middle comb could be removed first by pulling on its protruding top without mechanical complications, and this facilitated the removal of the other combs without damaging the sample wells.

**Comparison of performance by different gel matrices**

The CNG system demonstrated overall superior performance compared to that of polyacrylamide gels in resolving proteins of ultra-large molecular sizes such as the 12-subunit yeast RNA polymerase II, a protein complex with a molecular weight over 500 kDa and responsible for the synthesis of pre-mRNA in eukaryotes [19]. As shown in Fig. 2, RNA Pol II and GST–CTD (fusion protein containing GST protein and the C-terminal repeat region from the Rpb1 subunit of Pol II) showed smearing of bands in a 4% acrylamide gel (lanes 1 and 4, respectively), while this smearing reduced considerably upon electrophoresis using composite gels containing 0.5% agarose and 2% acrylamide (lanes 2 and 5, respectively). Remarkably, both of the proteins migrated as defined bands in CNG made with the SeaKem Gold grade agarose from Cambrex (lanes 3 and 6). While three different batches of the SeaKem Gold agarose performed indistinguishably, agarose from other commercial sources tested by us did not perform as well (data not shown). As agarose, which mainly contains the polysaccharide agarobiose, also contains a charged polymer agaropectic that slightly differs in the basic repeat unit, varying grades of purity are obtained depending on the degree of removal of this agaropectic [20,21]. Therefore, we think that the impurity present in the various grades of agarose may have resulted in trailing and blurring of the bands, possibly due to its interaction with the protein molecules. The conditions for an optimized CNG are given in Table 1. As we tested, variations of the conditions could be tolerated with the lower limits on acrylamide and agarose being 1.5 and 0.3%, respectively.

**Minimizing staining background in composite gels**

Different staining protocols have been tested for the CNG system to achieve lower background and thus higher contrast. The regular Coomassie blue stain used for SDS–PAGE (0.25% Coomassie blue R-250 in 40% methanol and 10% acetic acid) [22] proved inadequate
for staining the composite gel. The background persisted even after extensive destaining (Fig. 3, lanes 1–4). Remarkably, staining with Bradford reagent [23] produced significantly higher band-to-background contrast (Fig. 3, lanes 6–9), possibly due to the colloidal nature of Coomassie blue dye in Bradford reagent. Moreover, the gels stained with the Bradford reagent could be conveniently destained with distilled water. We are not certain of the reasons for the insufficient destaining of the regular Coomassie-blue-stained gels with the regular destainer containing methanol and acetic acid. We have observed minor diffusion of protein bands during the initial period of staining by Bradford reagent. This, however, could be avoided by rapidly fixing the gels with 10% trichloric acetic acid or methanol–acetic acid mixture, prior to staining. The composite gels after staining with Bradford reagent were completely amenable to quantification with densitometry.

Finally, the CNG proved to be compatible with regular silver staining [24] which is not quantitative but is 5- to 10-fold more sensitive than Coomassie-based staining in our hands (data not shown).

**Coupling CNG electrophoresis to second-dimension SDS–PAGE**

Coupling of native gel electrophoresis to a second-dimension denaturing gel electrophoresis is often necessary to verify and/or identify individual components within a complex that has been separated over the first dimension. We found that protein–protein interactions often resulted in an altered mobility in the CNG electrophoretograms. Complex formation also resulted in an increase in the intensity of the corresponding protein band. For instance, the formation of Pol II–Fcp1 complex led to the emergence of a band that migrated faster than Pol II alone but slower than the free Fcp1 protein (Fig. 3, lanes 6–8). Concomitantly, the intensity of the complex band (in lanes 6 and 8) also enhanced significantly over the free polymerase (lanes 7 and 9) even though the same amounts of Pol II were used, indicating an increase in the mass due to Fcp1 binding. However, in situations where the interaction does not produce a change in mobility (due to unaltered charge–mass properties) and/or does not increase staining intensities (due to a limited increase in mass), additional analysis becomes necessary. One such example is the interaction of λQ, a transcription antiterminator encoded by bacteriophage λ [25,26], with *E. coli* RNA polymerase (RNAP). When λQ was incubated with the holo or core RNAP, neither complex showed any significant change in mobility as compared to the free RNAPs (Fig. 4A, lanes 1–4). In addition, as λQ was known to migrate toward the cathode due to its overall positive charges, it was not clear whether the protein complex in lanes 3 and 4 contained λQ. To resolve this issue, we performed a second-dimension SDS–PAGE analysis [15,16] and the results presented in Fig. 4B clearly reveal the presence of λQ protein in both complexes. Noteworthily, we eliminated the usual step of soaking with SDS and β-mercaptoethanol for denaturation, therefore preventing protein loss due to diffusion into the soaking solution.

**CNG electrophoretic analyses of Pol II phosphorylation state and Pol II–Fcp1 complex**

Rpb1, the largest subunit of yeast RNA polymerase II, possesses a CTD that consists of a repeated heptapeptide motif, Tyr1-Ser2-Pro3-Tyr4-Ser5-Pro6-Ser7 [27,28], which can be phosphorylated at the serine residues at positions 2 and 5. Over the years it has been generally recognized that the CTD cycles between hyperphosphorylated (Pol IIo) and hypophosphorylated (Pol II) forms [29–32], while the polymerase with hypophosphorylated CTD is either free or at early stages of transcription leading to initiation [33–36]. Functional studies also indicate that CTD phosphorylation/
dephosphorylation events play key roles at the various stages of gene transcription in eukaryotes [37,38]. Therefore, methods for detecting CTD phosphorylation is of great importance in many experiments designed to study transcription mechanisms. To date, means of documenting CTD phosphorylation have relied solely on the change of Rpb1 mobility in SDS–PAGE or on immunoblotting with phospho-CTD-specific monoclonal antibodies. Both of these approaches, involving protein denaturation, are obviously not suitable for forming Pol II complexes with regulatory factors and analyzing the results simultaneously. We have employed the CNG system to distinguish between the two forms of Pol II. The result clearly shows a significant difference between the electrophoretic mobility of Pol II and that of Pol IIo (Fig. 5, lanes 2 and 3).

We have also used the CNG system to study the complex formation between RNA polymerase II and Fcp1, a CTD-specific phosphatase that functions to convert Pol IIo back to Pol II for polymerase recycling and transcription reinitiation [39–42]. We used the wild-type Fcp1 protein and a point mutant (D172N) in these studies. Pol II was incubated with either the wild-type Fcp1 or Fcp1(D172N) and the mixture subjected to CNG electrophoresis. As already shown in Fig. 3, a band corresponding to a complex between Pol II and Fcp1 emerged at a position clearly distinct from those of either free Pol II or Fcp1 (Fig. 5, lane 7). The same complex was also observed with the D172N mutant (Fig. 5, lane 8). To our knowledge, these data present the first direct evidence of a physical interaction between Pol II and Fcp1. In a related study, we have found that the interaction between Pol II and Fcp1 was dynamic enough to not allow the isolation of a stable complex.

Fig. 4. CNG–SDS two-dimensional electrophoresis of RNAP–λQ complex. (A) E. coli holo or core RNAP (5 μg each) was mixed with the transcription antiterminator Q from phage λ (λQ) (1 μg) and resolved over a CNG (2% polyacrylamide, 0.5% SeaKem agarose) in the first dimension (lanes 3 and 4). Free holo RNAP (Holo) and core RNAP (Core) were run in lanes 1 and 2, respectively. The native protein marker (MK) was in lane 5 with its components indicated on the right-hand side. No discernable change in gel mobility was observed for the RNAP–λQ complexes (Holo–λQ and Core–λQ). (B) The CNG strips corresponding to the holo RNAP–λQ (Holo–λQ) and the core RNAP–λQ (Core–λQ) complexes were each transferred to second-dimension SDS gels (10%), in the way described in the legend to Fig. 1. The arrowed bars on top of each SDS gel indicate directions of the first-dimension CNG electrophoresis. Identities for each protein subunit of RNAP are marked along the center (β, β′, α, and ω).

Fig. 5. Resolving various RNA Pol II complexes in CNG electrophoresis. (Left) Pol IIo, hyperphosphorylated on its CTD (lane 1), migrated significantly faster than the unphosphorylated Pol II (lane 2). MK is the native protein marker whose components are indicated along the center (Tg, Fer, and Cat). (Right) Pol II–Fcp1 and Pol II–Fcp1(D172N) complexes migrated significantly faster (lanes 7 and 8, respectively) than free Pol II (lane 2) but more slowly than the free Fcp1 and Fcp1(D172N) proteins (lanes 5 and 6, respectively).
using size-exclusion chromatography (M.-H. Suh and J. Fu, unpublished). However, the CNG method presented here was capable of capturing and revealing such types of weak and dynamic protein–protein interactions.

Interestingly, the CNG was sensitive enough to produce a small yet definitive difference between the migration of the wild-type Fcp1 and that of Fcp1(D172N) which lost only one negative charge compared to the wild-type protein at the pH value (8.0) of the gel buffer (Fig. 5, lanes 5 and 6). We have also examined a similar Fcp1 mutant, D170N, which also migrated at the same rate as D172N did (data not presented). Since no special reagents or conditions other than the buffer components have been required, it is obvious that the CNG system can also be used for studies of other multiprotein complexes with molecular weights greater than 500 kDa.

Variance and reproducibility of CNG

To assess the robustness of the CNG, we analyzed migration distance as a function of run duration. Strict linearity was observed for each of the three different proteins contained in the native protein marker kit (Fig. 6). In this set of experiments, 12 composite gels were cast, and each was run for a specified length of time. The resulting migration of each protein was plotted against the running time. As can be seen from the different slopes, resolutions of the proteins increase as the running time extends. Since each gel was prepared individually at different times, slight variations in concentrations of acrylamide and/or agarose were inevitable among the gels, yet variance among the data points was negligible, yielding a straight line for each of the proteins. We therefore conclude that the CNG procedure is reproducible using the gel assembling steps described under Materials and methods.

Discussion

Native gel electrophoresis is a valuable method for identifying and analyzing multiprotein assemblies, and it has the potential to be utilized more widely to address macromolecular complexes of excessive molecular masses. The usefulness of native electrophoretic analysis is well demonstrated by the blue native polyacrylamide gel electrophoresis (BN-PAGE), a modified version of the technique developed to suit the separation of membrane proteins [15,16] such as ATP synthase. Applications of that technique have led to the identification of dimeric forms of F1Fo–ATP synthases [43,44]. The BN-PAGE approach has since been generalized for proteomic characterization of membrane protein complexes [45]. This method utilizes the property of hydrophobic and nonspecific binding of Coomassie dye molecules to membrane proteins, thereby conferring net negative charges to the protein molecules [46]. BN-PAGE usually is operated with gels of about 4% polyacrylamide. However, from our experiments with yeast RNA polymerase II and its regulatory factors, the BN-PAGE technique proved inadequate for soluble proteins and often resulted in very limited distances of migration (J. Fu, unpublished). Conceivably in theory, lowering acrylamide percentage should allow better migrations, but gel-handling problems associated with gels lower than 4%, again, impede the application in slab configurations.

Using the agarose–polyacrylamide composite native gel system, we have found a way to circumvent the operational difficulties associated with low-percentage (≤2%) polyacrylamide gels. The CNG is capable of providing a sturdy physical support at very low polyacrylamide percentages (e.g., 1.5%) with satisfactory electrophoretic performance. The mechanical strength of the CNG comes from the integrated framework of agarose polymers. Since agarose is supplied at very low concentrations (e.g., 0.5%) here, it forms only a loose meshwork in the composite gels, while polyacrylamide forms the main resolving matrix.

We have experimented with conditions to find the physical limitations on forming the CNG. We found that composite gels composed of less than 1.5% acrylamide with 0.3% agarose did not yield good resolutions for the Pol II protein and its complexes with transcription factors, leading to smearing of protein bands. On the other hand, higher percentages of acrylamide (3–4%) tended to hinder the entry of such large protein complexes into the
gel matrix. We have also found 0.3% to be the lowest agarose percentage that could be used to provide sufficient mechanical strength. We conclude that CNG with 2% acrylamide and 0.5% agarose is the optimum for protein studies at pH 8.0. Of course, the pH value of a running system can be adjusted to suit the electrostatic-charge status of target proteins. We have also investigated the cooling step (required for prior gelation of agarose) with different cooling temperatures. In our experience, cooling at 4 °C performed best. Chilling in icy water was too drastic, resulting in gel shrinkage, although it was used in a previously reported method for making denaturing hybrid gels [9]. We have demonstrated that, when these steps were followed closely, the CNG procedure was highly reproducible with negligible variations between different gel runs (Fig. 6).

The CNG described here provides an effective means to characterize supramolecular complexes of the Pol II transcription machinery. To begin with, it could clearly distinguish the hyperphosphorylated Pol IIo from the unphosphorylated Pol II (Fig. 5) under native conditions, thereby providing a method that can be used to study complex formation between either forms of Pol II with regulatory factors. Binary, ternary, and even higher-order complexes can be subjected to the CNG electrophoresis and discrete entities may be identified. As a first demonstration of the effectiveness of this technique, we obtained distinctive separation of Pol II–Fcp1 complex from either the free Pol II or the free CTD phosphatase (Figs. 3 and 5). Of particular interest is the stabilization effect from the gel that allowed this complex to be captured and revealed. The interaction between Pol II and Fcp1 exhibited a dynamic behavior in a related study that included various assays such as gel-filtration chromatography and GST pulldown (J. Fu and M.-H. Suh, unpublished). This stabilization of complex formation is consistent with the known fact that gel matrices impose “caging” effects, thereby increasing the local concentration of macromolecules. In addition, the CNG system is highly sensitive as it has been able to produce a definitive migration difference between the wild-type Fcp1 and the Fcp1(D172N) (Fig. 5), which differ by only a single negative charge per 82 kDa of molecular mass. These merits are not attainable from methods such as gel-filtration chromatography (or SEC) and analytical ultracentrifugation, which separate molecular species solely by hydrodynamic properties. For instance, SEC is suitable mainly for the analysis of stable complexes but not for the characterization of relatively week or dynamic complexes such as Pol II–Fcp1, because the separation involves continuous partitioning of the components over extended time periods.

We have also demonstrated the practicability of coupling the CNG electrophoresis to a second-dimension SDS–PAGE to facilitate the identification of individual subunits within each protein entity separated over the CNG. This scheme worked efficiently for the complexes of both E. coli RNAp and yeast Pol II used in this work and proved critical in identifying the complex formation between E. coli RNAp and λQ (Fig. 4). We have applied this technique to the characterization and verification of a great number of Pol II–factor(s) complexes (M.-H. Suh and J. Fu, unpublished) and found this method highly effective and convenient. Since no specific reagents such as detergents or dye molecules are required, the CNG electrophoresis described here will be applicable to other multiprotein systems where excessive molecular masses are involved.

**Acknowledgments**

We thank Drs. Stewart Shuman (Sloan-Kettering Institute), Averell Gnatt (U. Maryland Schl. Med.), and Jeffrey Roberts (Cornell U.) for sharing reagents. Work presented here has been supported in part by a grant from NIH (GM064651) to J.F. and an institutional grant from American Cancer Society (IRG-01-231-01).

**References**


[34] P.J. Laybourn, M.E. Dahmus, Phosphorylation of RNA polymerase IIA occurs subsequent to interaction with the promoter and before the initiation of transcription, J. Biol. Chem. 265 (1990) 13165–13173.


