The Fate of the Nucleosome During Transcription by RNA Polymerase II

By WALTER, W., KIREEVA, M.L.*, TCHERNAJENKO V., KASHLEV, M.* and STUDITSKY V.M.†

Department of Biochemistry and Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, MI 48201

* NCI Center for Cancer Research, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD 21702-1201

† To whom correspondence should be addressed

RUNNING TITLE: Analysis of Nucleosomes Transcribed by RNA Pol II

1.0 Introduction

Eukaryotic genes transcribed by RNA polymerase II (Pol II) retain nucleosomal structure [1]. Thus, Pol II encounters nucleosomes every ~200 bp during transcript elongation *in vivo*. The mechanism of transcription through chromatin and the regulation of this process are poorly studied. Until now, only two types of Pol II-based experimental systems *in vitro* were available for analyzing the outcome of a Pol II-nucleosome encounter [2-4]. One type includes systems supporting promoter-dependent transcription initiation, either in crude extracts [3] or with highly purified proteins [4]. The main disadvantage of this approach is that only ~1 % of the templates are transcribed [5]. This low efficiency of template utilization makes analysis of the fate of nucleosomes after transcription nearly impossible. In contrast, DNA templates containing a single-stranded, 3'-extending oligo dC "tail" support efficient end-initiation by Pol II *in vitro* [2]. However, in this system, determination of the fate of the nucleosome during transcription is complicated by the formation of extremely stable DNA-Pol II complexes at the end of DNA [6]. Moreover, it is likely that end-initiated and promoter-initiated RNA polymerases differ in the way they progress through the nucleosome [6].

More recently, a novel approach for analysis of Pol II elongation complexes (ECs) was developed [7,8]. It employs assembly of "authentic" ECs using histidine-tagged yeast Pol II and synthetic RNA and DNA oligonucleotides. The structure and functional properties of the assembled and promoter-initiated ECs are very similar [8,9]. In this system, the fate of nucleosomes during transcription can be analyzed after ligation of the ECs to positioned mononucleosomes that are assembled separately [10]. Nucleosomes form an absolute block to Pol II, but this barrier can be reduced by increasing the ionic strength of the reaction, thus allowing more templates to be

transcribed. Transcription through nucleosomes by Pol II induces the loss of an H2A/H2B dimer without changing the position of the histones on the DNA [10].

Use of this experimental system for analysis of the fate of the nucleosome during transcription is described below. This "minimal" model system can be easily adopted for analysis of polynucleosomal templates and for analysis of the role of different elongation factors during transcription through chromatin. Similar approaches can be applied for analysis of a variety of biological processes (such as chromatin remodeling, DNA replication, recombination, and repair) that may involve changes in nucleosome positioning or the histone content of nucleosomes.

2.0 Materials and Methods

I. Template Preparation

Purification of core histones and donor chromatin

The detailed protocols for purification of core histones on hydroxyapatite [11,12] and purification of donor chromatin [13,14] were published and will not be discussed here.

Template Design and Purification of DNA

Careful consideration should go into the design of the template to be used for analyzing transcription through the nucleosome by Pol II. First, the ECs and nucleosomes are assembled separately and then ligated together. Thus, for efficient ligation, a long, asymmetric sticky end, such as that generated by TspRI cleavage, is preferable. Second, a defined template with a positioned nucleosome(s) is desirable for mechanistic studies. For this, strong and well characterized nucleosome positioning sequences, such as that of the *Xenopus* 5S RNA gene can be employed [10,15]. Finally, to map nucleosome positioning before and after the reaction, there

should be several restriction enzyme sites along the entire length of the template for restriction endonuclease protection assays.

The pVT1 Template [10]

The template DNA is PCR amplified from pVT1 plasmid by using the primers (Invitrogen Corporation, Carlsbad, CA) pVT1-431-454-up (5' GAC ACT ATA GAA TTA ATG GGG ATC 3') and pVT1-737-716-low (5' CCT TCC AAG TAC TAA CCA GGC C 3'). Some of the lower primer is radiolabeled with $\gamma[^{32}P]$ATP (7000 Ci/mmol, ICN Biomedicals, Inc., Irvine, CA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, as per NEB recommendations) prior to the PCR. The resulting 306 bp product is digested with TspRI, and the sample is loaded onto an 8% (19:1) polyacrylamide gel containing 1X TAE and 4 M urea (to prevent re-association of the 9 nt, GC-rich sticky ends of the TspRI-digested fragments). The 204 bp TspRI-StuI fragment is cut out of the gel, the gel slice is crushed, and the DNA is extracted overnight at 4^0C in 3-5 volumes of TE buffer, ethanol precipitated, and resuspended in dH_2O. The template DNA is further "cleaned up" using QIAquick gel extraction kit columns (Qiagen, Chatsworth, CA, as per kit protocol).

Reconstitution, characterization, and purification of mononucleosomes and hexasomes

It is desirable to find efficient reconstitution conditions where the amount of free DNA is minimal (<10-15%) so the nucleosomes do not have to be further purified. The methods described below are for templates that are about 150-250 bp in size and, thus, allow for only one nucleosome per molecule of DNA.

Reconstitution of mononucleosomes via octamer exchange from donor chromatin

One method of making nucleosomal templates is to use donor chromatin as a source of histone octamers for exchange [14] onto a template of interest. This method was chosen to reconstitute nucleosomes for transcription because it is generally very efficient (the amount of free DNA is <15%), and nucleosomes do not have to be purified. Nucleosomes prepared this way contain excess donor chromatin, but it can be removed by washing after the template is ligated to immobilized ECs.

DNA (1-5 μg) is mixed with long -H1 donor chromatin at a ratio of 1:60 (wt:wt), respectively, sample volume is determined by donor chromatin concentration) in buffer containing 1 M NaCl and 0.1% Igepal CA-630 (Sigma, St. Louis, MO). The sample is dialyzed overnight at 4^0C against a gradient (~1 L) starting at 1 M NaCl and ending with no NaCl in buffer containing 10 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, and 0.1% NP-40.

Reconstitution of mononucleosomes and subnucleosomal particles from purified histones

Nucleosomes can also be reconstituted from purified histones. The protocol described below is a slightly modified version of the method used by the Bradbury laboratory [16,17]. This procedure can also be employed to create subnucleosomal particles by varying the amount of H2A/H2B used.

To make nucleosomes, 5 μg of DNA is mixed with 1.23 μg of H3/H4 and 2.70 μg of H2A/H2B (ratio of H3/H4:H2A/H2B= 0.455) at a volume of 100 μl in buffer containing 2 M NaCl, 0.2 mM EDTA, 10 mM Tris-HCl (pH 7.4), and 0.1% Igepal CA-630 (Sigma, St. Louis, MO). To make hexasome, the amount of H3/H4 is kept constant while the amount of H2A/H2B is reduced to 1.35 and 0.67 μg (with ratios of H3/H4:H2A/H2B= 0.91 and 1.82, respectively).

Tetrasome is reconstituted by using the same amount of H3/H4 but eliminating the H2A/H2B. Dialysis is performed at 4^0C against the same buffer but with decreasing NaCl concentration (2 M, 1.5 M, 1 M, 0.75 M, 0.5 M, and 10 mM NaCl) for 1 hour at each step.

Characterization and purification of nucleosomes and chromatin subparticles

Nucleosome and hexasome preparations must be analyzed for the amount of free DNA, hexasome, and nucleosome present in each preparation as well as the location of histones on the template. The position of a nucleosome can be determined with about 10 bp resolution based on its mobility during native PAGE [17]. However, this method cannot discriminate between two symmetrically positioned nucleosomes or differently positioned nucleosomes formed on DNA ~200 bp or less [17]. The exact position can be further narrowed down by restriction enzyme digestion or micrococcal nuclease mapping (not discussed here [18]).

For analysis, the reconstitutes (10 ng aliquots) are supplemented with buffer providing 20 mM Na-HEPES (pH 7.8), 5 mM $MgCl_2$, 2 mM spermidine (Sigma, St. Louis, MO), and 0.5 mg/ml BSA. One sample is not digested, while appropriate restriction enzymes (10 U) are added to the others, and digestion is performed at room temperature (RT) for 0.5-1 hour. Buffer is added providing a final concentration of 10 mM EDTA, 10% sucrose, and 250 μg/ml sheared herring testes DNA (Intergen, Purchase, NY), and the templates are resolved by native gel electrophoresis (4.5% acrylamide (39:1), 5% glycerol, 20mM Na-HEPES (pH 8), 0.1 mM EDTA) at 100 V for 2.5-4 hrs (depending on the size of the DNA fragment and the degree of resolution desired) as described [18]. Quantitation is performed using a Cyclone Storage Phosphor System (Packard, Meriden, CT). If gel purification is required [18], the samples are loaded without carrier DNA. The appropriate band is cut out of the gel, the gel is crushed, and the DNA,

nucleosome, or hexasome are extracted overnight at 4^0C in 1-2 volumes of 10 mM Na-HEPES (pH 8.0), 0.1 mM EDTA, and 0.5 mg/ml BSA. The supernatant is collected, and the concentration of the sample is determined by the specific activity of the DNA.

Analysis of the pVT1 Reconstitutes [10]

A. Nucleosomes formed *via* octamer exchange

Nucleosomes are reconstituted very efficiently on the 204 bp (TspRI-StuI) pVT1 template by octamer exchange (Figure 1, lane 1). There is one nucleosomal band, but the templates are too short to be resolved based on nucleosome position. Digestion with EcoRI (lane 2) and EcoRV (lane 3) and EcoRI+EcoRV (not shown) reveals that about half of the nucleosomes are resistant to EcoRI and sensitive to EcoRV (N1) and half are resistant to EcoRV and sensitive to EcoRI (N2). The pVT1 DNA contains the strong 5S nucleosomal positioning sequence, so one nucleosome position (N2) was expected. However, the TspRI end of the DNA is an equally good site for nucleosome formation (N1).

B. Nucleosomes and hexasomes reconstituted from purified histones

Nucleosomes are not reconstituted as efficiently from purified histones as they are with exchange (compare Fig. 2A, lane 1 and Fig. 1, lane 1). Restriction enzyme digestion with EcoRI and EcoRV reveal that nucleosomes formed by this method are indistinguishable from those formed by exchange. As the amount of H2A/H2B is decreased, less nucleosome is formed and more hexasome is present in the sample (Fig. 2A).

Due to the large amount of free DNA in the samples, the nucleosomes and hexasomes have to be gel purified (Fig. 2B). The yield for this procedure is extremely low. Therefore, this was not the protocol of choice for preparing nucleosomes used for transcription.

II. Transcription of defined nucleosomal templates

Transcription Buffers

TB0 contains 20 mM Tris-HCl, pH (7.9), 5 mM $MgCl_2$, and 1-2 mM β-mercaptoethanol (2-ME). TB40, TB150, TB300, TB700, and TB1000 contain 40 mM, 150 mM, 300 mM, 700 mM, and 1 M KCl, respectively. Acetylated BSA was purchased from Sigma (St. Louis, MO).

EC assembly and ligation to DNA or nucleosomal templates

Yeast Pol II ECs are assembled from purified components as described in [19], using the following oligonucleotides (*Oligos, Etc*. Inc., Wilsonville, OR): TDS50 (5' GGTGTCGCTTGGGTTGGCTTTTCGGGCTGTCCCTCTCGATGGCTGTAAGT 3'), RNA9 (5' AUCGAGAGG 3'), and NDS59 (5' ACTTACAGCCATCGAGAGGGACACGGCGAAAA GCCAACCCAAGCGACACCGGCACTGGG 3'). The TDS50 oligo is labeled with γ[^{32}P]ATP (7000Ci/mmol, ICN Biomedicals, Inc., Irvine, CA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) as described [19]. The Ni^{2+}/NTA agarose (Qiagen, Chatsworth, CA) is pre-treated with 0.2 mg/ml acetylated BSA for 10 min and washed 2X with 1 ml of TB40 prior to immobilization of Pol II.

ECs assembled with ~2-3 pmol of Pol II (immobilized on 50 µl of 50% resin suspension, enough for ~10-15 reactions) are incubated in the presence of 100-200 ng of template, 100 µM ATP, 1% PEG-8000, and 50 units of T4 DNA ligase (New England Biolabs, Beverly, MA) in a

volume of ~100 μl at 12^0C for 1-2 hours. The ECs are washed with TB40, incubated for 10 min with TB700, and washed twice with TB40. The ligation efficiency achieved by using the 9 nt 3' overhang generated by TspRI cleavage (5' CCCAGTGCC 3') is at least 50%.

Analysis of ECs and nucleosome mapping after ligation

ECs (assembled with ~1-1.5 pmol of Pol II, ligated to nucleosomes and washed) are eluted in a volume of ~50 μl in TB40 containing 0.5 mg/ml acetylated BSA and 100 mM imidazole (Sigma, St. Louis, MO). The samples are incubated at RT for 10 min., diluted 2-fold, mixed, and microcentrifuged for 10 sec. 50 μl of the supernatant is withdrawn and diluted 5-fold with buffer providing 20 mM Na-HEPES (pH 7.6), 5 mM $MgCl_2$, 2 mM spermidine (Sigma, St. Louis, MO), 0.5 mg/ml acetylated BSA, and 1 mM β-mercaptoethanol. The sample is aliquoted (10 μl each), restriction enzyme digestions (with 10 U of enzymes) are performed for 30-60 min at RT, and the products are analyzed in a native gel as described above.

Analysis of ECs after ligation to the pVT1 nucleosomal template and mapping of the ligated nucleosomes.

Nucleosome positioning on the pVT1 template was analyzed after ligation to labeled EC9 (EC9 denotes an elongation complex with RNA that is 9 nt long) and elution with imidazole. Note the faster mobility of the unligated EC9 as compared to the EC9 ligated to the nucleosomal template (EC9N, Fig. 3B, lane 1). Some of the ECs (5-10%) spontaneously dissociate during elution and electrophoresis, and the free nucleosomes (N1 and N2) are resolved based on their positions along the DNA. As expected, digestion with EcoRI and EcoRV (lanes 2 and 3) reveals

that that about half of the templates (Pol II-bound or not) are sensitive to EcoRI and half are sensitive to EcoRV. A detailed map of the templates used for transcription is shown in Fig. 3A.

Transcript elongation

Immobilized Pol II can be walked to any point along the DNA by simply adding a subset of NTPs to the reaction, washing the resin, adding a different subset of NTPs, and so on. The concentration of NTPs added and the length of incubation vary based on the template, the location of the EC on the DNA, and the NTPs used [20]. The conditions described below are specific for the pVT1 template.

The nucleosome is an extremely strong barrier for Pol II under physiological conditions. This makes analysis of the transcribed templates difficult as most of the polymerase is stuck in the nucleosome, and the templates are not released. However, at 300 mM KCl, ~30% of the polymerase can overcome the barrier and finish transcription through the nucleosome [10]. Therefore, to analyze the fate of the nucleosome after transcription, the reaction is carried out at 300 mM KCl so that enough templates are transcribed and released for analysis.

It is important to analyze only those templates that are fully transcribed and released by the polymerase. However, it is common for ECs to become arrested during transcription of the first 15-50 nt. Many of these ECs dissociate, and this results in contamination of the supernatant with non-transcribed templates. For this reason, Pol II is walked to the +45 position on the pVT1 template, and the ECs are washed extensively to remove any non-transcribed templates.

Walking Pol II on the pVT1 template to form EC45

The first 45 nt of the pVT1 template consist of a -UTP cassette (see Fig. 3A). Thus, in the absence of UTP, Pol II can be walked to the +45 position (EC45). The volume of the EC9N (assembled with ~2-3 pmol of RNAP) is adjusted to 100 µl (enough for 10-10 µl reactions) in TB40 containing 0.2 mg/ml acetylated BSA. The ECs are incubated in the presence of 200 µM ATP, CTP, and GTP for 10 minutes at RT. The sample is mixed, microcentrifuged for about 10 sec, and 50 µl of the supernatant (S1) is withdrawn and divided into 5 µl aliquots. The S1 supernatant contains free templates as a result of early EC dissociation, and is representitive of non-transcribed templates. The pellet is washed 2 times with 1 ml of TB300, incubated in 1 ml of TB300 for 15 min, and washed 2 more times with 1 ml of TB300. This procedure is repeated to remove any remaining non-transcribed templates.

Immobilized transcription of the pVT1 template

Many controls are done to make sure that the templates analyzed are actually released as a result of transcription and not present due to further EC dissociation. The volume of the washed pellet (EC45) is adjusted to 100 µl in TB300 containing 0.5 mg/ml acetylated BSA. The sample is aliquoted (10 µl per experimental point) and incubated for 5 min at RT with (i) no NTPs (S2), (ii) 100 µM ATP, CTP, and GTP (S3), or (iii) 100 µM NTPs (S4). The samples are mixed, briefly centrifuged, and the supernatants (S2-4, 5 µl per experimental point) are withdrawn for analysis. Supernatant S2 is a control for EC dissociation without NTP addition, S3 is a mock-transcription control for EC dissociation in the presence of NTPs, and S4 contains the transcribed templates.

Transcription of the pVT1 template in solution

For transcription in solution, the volume of the washed EC45 is adjusted to 100 μl in TB300 containing 0.5 mg/ml acetylated BSA and 100 mM imidazole (Sigma, St. Louis, MO). The ECs are eluted for 10 min at RT. The sample is mixed, centrifuged, and 50 μl of the supernatant is withdrawn. The supernatant is diluted 5-fold with TB300 containing 0.5 mg/ml acetylated BSA. The EC45 is aliquoted (10 – 20 μl per experimental point) and incubated in the presence of (i) no NTPs (E2), (ii) 100 μM ATP, CTP, and GTP (E3), or (iii) 100 μM NTPs (E4) for 5 min at RT.

Analysis of the transcribed templates

For analysis, the samples (S1-4 or E1-4) are supplemented with buffer providing 20 mM Na-HEPES (pH 7.8), 5 mM $MgCl_2$, 2 mM spermidine (Sigma, St. Louis, MO), and 0.5 mg/ml BSA and aliquoted into several tubes. One sample of each fraction is not digested, while appropriate restriction enzymes (10 U) are added to the others, and digestion is performed at RT for 0.5-1 hour. Buffer is added providing a final concentration of 10 mM EDTA, 10% sucrose, 250 μg/ml sheared herring testes DNA (Intergen, Purchase, NY), and 10 ng of carrier nucleosomes (to block non-specific interactions with the wells of the gel, donor chromatin works well), and the templates are resolved by native gel electrophoresis as described above. Quantitation is performed using a Cyclone Storage Phosphor System (Packard, Meriden, CT).

Reconstituted and gel-purified 204 bp hexasome is ligated to annealed NDS59 and ^{32}P-labeled TDS50 to make a mobility control for the 254 bp hexasome. Ligation is performed by incubating equimolar amounts of the annealed TDS50 and NDS59 and the 204 bp hexasome in the presence of 100 μM ATP, 1% PEG-8000, and 50 units of T4 DNA ligase (New England

Biolabs, Beverly, MA) at 12^{0}C for 1-2 hours. The sample is aliquoted, digested, and prepared for electrophoresis as described above.

Results from analysis of transcription of the pVT1 nucleosome, both with immobilized Pol II and in solution, are shown in Figs. 4A and 4B, respectively. The transcribed template has the same mobility in a native gel as the hexasome control. Furthermore, the restriction enzyme digestion profile of the transcribed template and the reconstituted hexasome are very similar. Moreover, the digestion patterns (half sensitive to EcoRI and half sensitive to EcoRV) are similar between the templates before and after transcription. Thus, transcription by Pol II results in the loss of an H2A/H2B dimer, but the remaining histones stay in their original positions on the DNA.

3.0 Concluding Remarks

Controls

This section briefly describes some additional controls that are used for this procedure.

Analysis of RNA released after transcription

It can be demonstrated that only the fully transcribed templates are released into the S4 supernatant by analyzing the transcripts that are released into the supernatant. A small amount of smaller transcripts may be present (from EC dissociation), so the controls (S2 and especially S3) are important for determining the background amount of EC dissociation.

EC9 is assembled with γ-[^{32}P]ATP-labeled RNA9 and phosphorylated TDS50 and immobilized on acetylated BSA-treated Ni^{2+}/NTA agarose as described [19]. EC9 (from ~1.5 pmol Pol II) is ligated to the nucleosomal template as described above, and the volume is adjusted to

50 μl. 10 μl of resin is aliquoted into 1 tube, while 40 μl of resin is aliquoted into another tube. EC45 is formed in each tube by the addition of 200 μM ATP, CTP, and GTP for 10 min at RT in TB40 and 0.2 mg/ml acetylated BSA. For tube #1, 5 μl of the supernatant (S1) is withdrawn, and the pellet fraction (P1, also 5 μl) is kept for analysis. For tubes #2-4, the resin is washed with 1 ml of TB300 twice, incubated with 1 ml of TB300 for 15 min, and washed with 1 ml of TB300 twice again. This procedure is repeated, and the resin is adjusted to a volume of 40 μl in TB300 containing 0.5 mg/ml acetylated BSA. 10 μl of the resin is aliquoted into each of 3 tubes. 5 μl of the supernatants are withdrawn after a 5 min incubation with: (i) no additions (S2), (ii) 100 μM each ATP, CTP, and GTP (S3), and (iii) 100 μM NTPs (S4). The corresponding pellet fractions (P2-4, also 5 μl each) are also kept for analysis. An equal volume (5 μL) of gel-loading buffer containing 50 mM EDTA and 8 M urea is added, the samples are boiled for 5-10 min at 95^0C, and the RNA is analyzed in a 6% (19:1) denaturing polyacrylamide gel. Quantitative analysis is performed with a Cyclone Storage Phosphor System (Packard, Meriden, CT). Fig. 5 is an example of the results obtained with this procedure on the pVT1 nucleosomal template.

Analysis of templates released after transcription of free DNA

It is also important to transcribe the free DNA template exactly like the nucleosomal templates are transcribed. The procedures for ligation, transcription, and analysis of the DNA template are the same as those described above for the nucleosomal template.

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Figure Legends

Fig. 1. Analysis of nucleosomes reconstituted by octamer exchange. Nucleosomes formed on 204 bp DNA (labeled at the StuI end, indicated by a star) were loaded onto a native gel before and after digestion with EcoRI and EcoRV. Positions of the EcoRI and EcoRV sites on the template are labeled. Mobilities of the nucleosomes (N1 and N2), free DNA, and the products of their digestion are indicated at the right. Molecular weight markers are an MspI digest of pBR322 DNA.

Fig. 2. Analysis of nucleosomes and hexasomes reconstituted from purified histones. A) Nucleosomes and hexasomes formed on 204 bp pVT1 DNA. Mobilities of the nucleosomes (N1 and N2) and hexasomes (H1 and H2), and the free DNA are indicated at the right. **B)** Gel-purified nucleosomes (N) and hexasomes (H).

Fig. 3. Analysis of Pol II ECs and Nucleosomes After Ligation. A) A map of the pVT1 template. ECs are labeled at the TspRI site (star) and ligated to nucleosomes. The Pol II start site is indicated with an arrow. The first 45 nt to be transcribed by Pol II lack UTP. Positions of nucleosomes (N1 and N2) and restriction enzyme sites used for mapping are indicated. **B)** Imidazole-eluted ECs were analyzed in a native gel before and after EcoRI or EcoRV digestion. The upper panel is a shorter exposure of the gel. Mobilities of unligated EC (EC9) and EC ligated to the nucleosomes (EC9N1 and EC9N2) are indicated. The lower panel is a longer exposure of the gel and reveals the free nucleosomes, N1 and N2, and free DNA fragments (marked by dots).

Fig. 4. Analysis of transcribed pVT1 nucleosomal templates. Templates were labeled as in Fig. 3A. **A)** Templates transcribed by immobilized polymerase [21]. Samples were analyzed before and after digestion with EcoRI, EcoRV, or EcoRI + EcoRV. The 254 bp reconstituted hexasome control (H) is indicated. Mobilities of the original nucleosomes (N1 and N2), the hexasome, free DNA, and non-ligated promoter fragment are indicated. S1 is non-transcribed templates, S3 is the mock-transcribed control, and S4 is transcribed templates. **B)** Templates transcribed in solution. Samples were analyzed by their mobility in a native gel beside the 254 bp reconstituted hexasome control. Mobilities of the ligated EC45, non-ligated EC45, free nucleosomes (N1 and N2), hexasome, DNA, and non-ligated promoter DNA are indicated. An underexposure of the top of the same gel is shown in the upper panel. Active and arrested ECs are indicated.

Fig. 5. Analysis of EC stability and NTP-dependent release of RNA/DNA [21]. ECs with labeled RNA9 were ligated to nucleosomes, and EC45N was formed. Supernatant (S1-4) and pellet (P1-4) fractions were collected and loaded onto a 6% denaturing gel. The mobilities of the 45 nt RNA and the run-off product are indicated by arrows. Transcripts from arrested ECs are marked by shaded bars.

Fig. 1

Fig 2

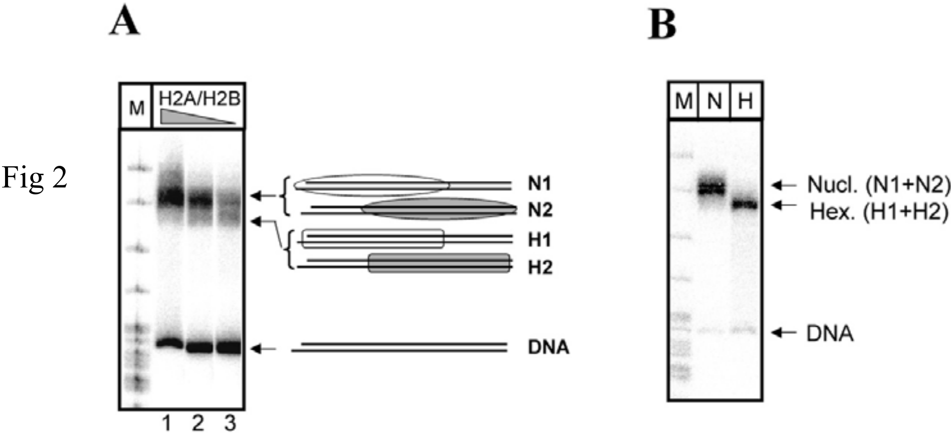

Fig. 3

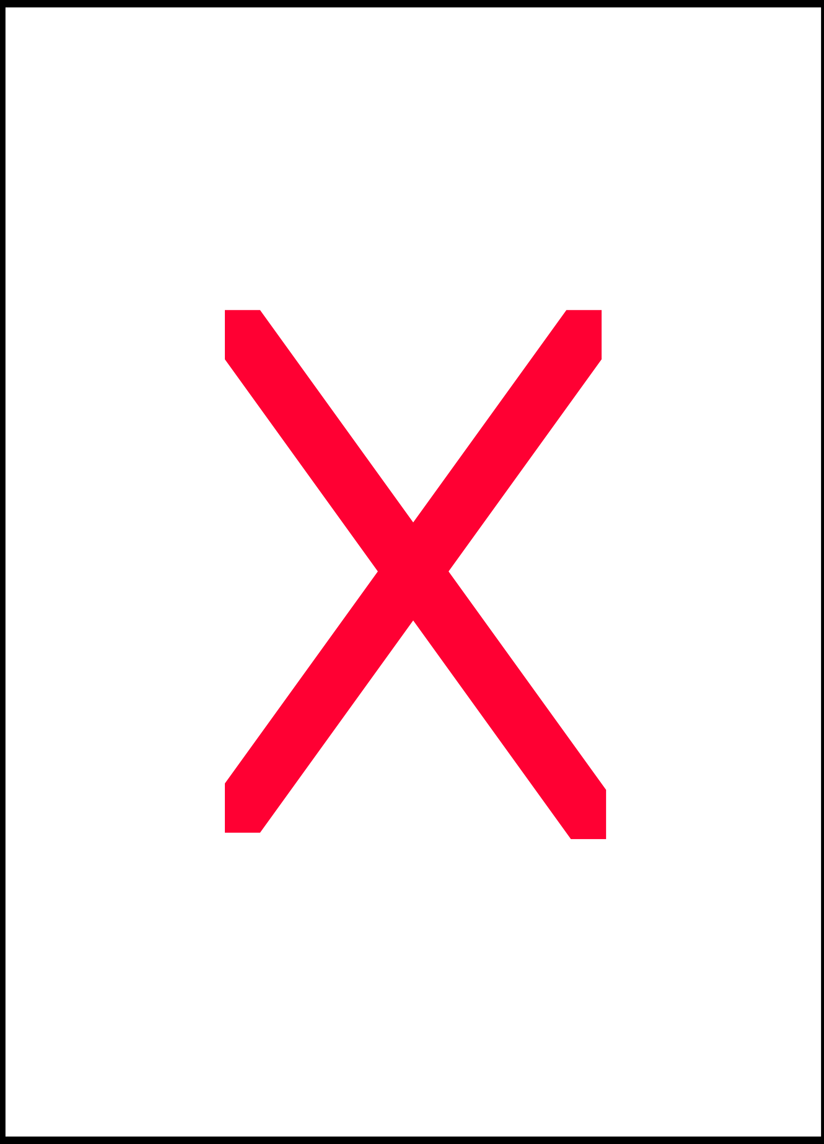

Fig. 4

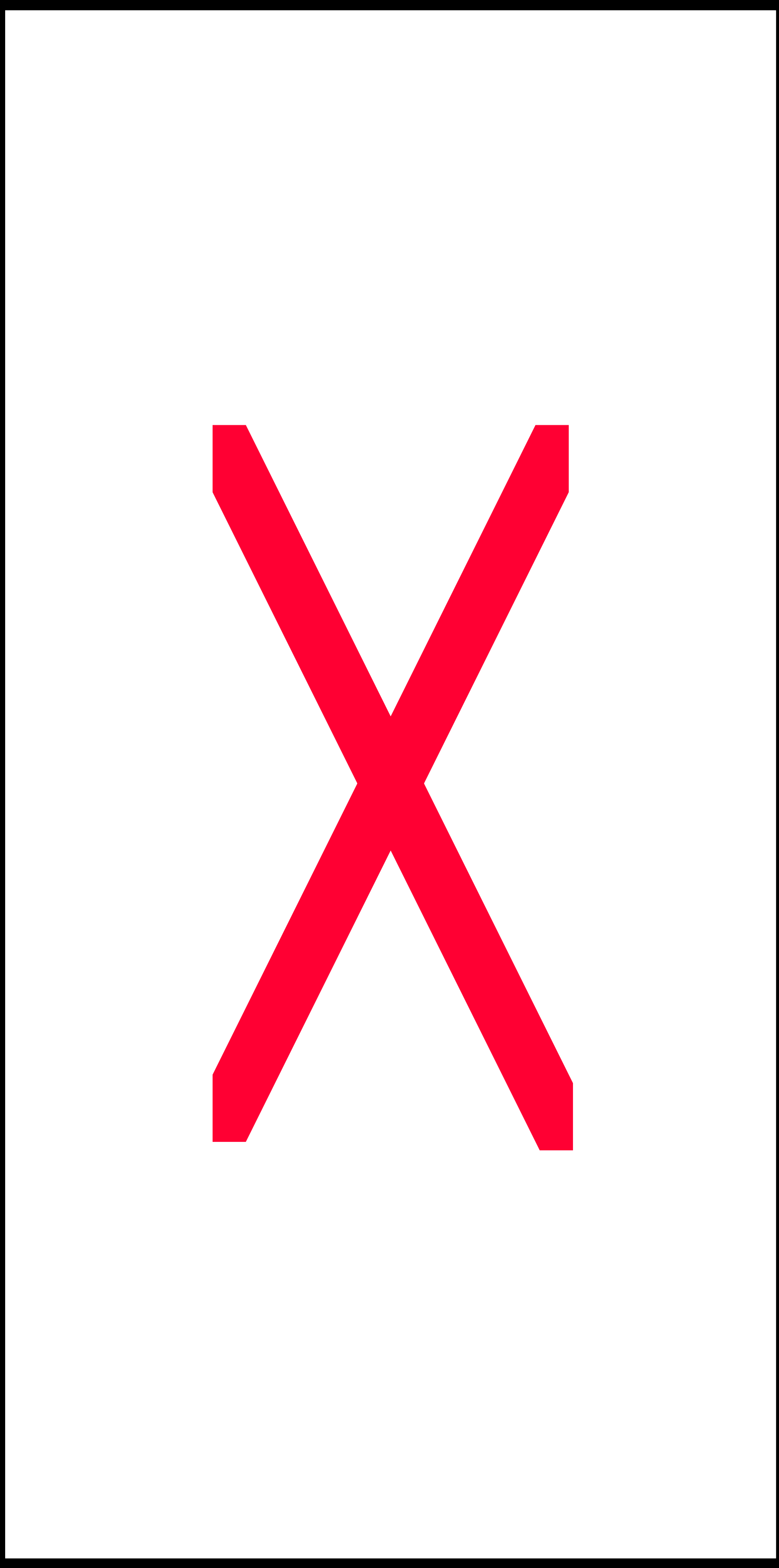

Fig. 5