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Nucleosome Assembly of Simian Virus 40 DNA in a Mammalian Cell Extract

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We report here a mammalian cell-free system that can support chromatin assembly. Effective nucleosome assembly in HeLa cell extracts occurred at 125 to 200 mM KCl or potassium glutamate. At this physiological K^+ ion concentration, two types of chromatin assembly were observed. The first was interfered with by Mg^{2+}. Other cations such as Mn^{2+}, Ca^{2+}, Fe^{3+}, and spermidine also inhibited this type of nucleosome assembly. The second type of assembly occurred in the presence of Mg^{2+} and at least equimolar ATP. However, even in the presence of ATP, excess Mg^{2+} inhibited assembly and promoted catenation of DNA; these effects could be circumvented by excess ATP, GTP, EDTA, or polyglutamic acid. The critical DNA concentration for optimum assembly in both pathways suggested a stoichiometric association of histones with DNA. The spacing of nucleosomes formed by both types of assembly on linear and circular DNA was reasonably regular, but chromatin assembled in the presence of ATP and Mg^{2+} was more stable.

The *Xenopus laevis* oocyte extract is the most extensively studied physiological system available for in vitro chromatin assembly. The lack of an appropriate mammalian cell-free system has forced investigators to transfer chromatin assembled in *Xenopus* extracts to a homologous mammalian cell extract to achieve RNA polymerase II (pol II)-specific transcription (33, 42, 65). The potential limitations of such heterologous systems underscore the need to develop mammalian cell-free systems that can support physiological chromatin assembly and pol II-mediated transcription of homologous genes.

Numerous previous in vitro nucleosome assembly studies involved either reconstitution of purified core histones and naked DNA (24, 25, 54) or assembly of DNA in cell extracts (25, 35, 44, 45, 52). The systems and experimental conditions were diverse, and the conclusions arrived at showed serious inconsistencies. For example, assembly in a *Drosophila melanogaster* extract (45) produced a periodic organization of nucleosomes. However, when a *Xenopus* extract was used, periodic nucleosome spacing was observed only when reconstitution was done in the presence of ATP and Mg^{2+} (52). DNA assembled with purified core histones had an irregular spacing of nucleosomes (34) except when reconstitution was done in the presence of poly-L-glutamic acid (54). Nucleosome assembly in a HeLa cell extract capable of supporting RNA synthesis was very inefficient (44), and unlike in *Xenopus* oocytes, it was not promoted by the presence of ATP and Mg^{2+} (52). Moreover, chromatin assembly occurred preferentially on replicating DNA in a human-cell nuclear extract (56).

Our long-term objective is to study chromatin assembly and its relationship to in vitro transcription mediated by pol II. We chose simian virus 40 (SV40) DNA and a HeLa cell extract. Circular SV40 DNA has been a convenient system with which to study nucleosome assembly (23, 36). Moreover, one can measure RNA synthesis from the assembled chromatin, since SV40 DNA contains well-characterized pol II-transcribed genes. HeLa cell extracts are most commonly used to study pol II transcription in vitro. There is one major disadvantage with the HeLa cell extract: added cofactors and topoisomerase II present in this extract produce extensive catenation of circular DNA, the substrate conventionally used to measure nucleosome assembly (36).

In pilot studies, we noticed that catenation of circular DNA by topoisomerase II in the presence of ATP and Mg^{2+} was much faster than nucleosome assembly in the absence of cofactors. Thus, a system potentially capable of assembling DNA into nucleosomes would catenate that DNA long before nucleosomes could assemble completely. To circumvent this problem, we sought to minimize the ATP and Mg^{2+} concentrations by using an ATP-regenerating system. A systematic variation of other biochemical parameters, including ionic strength and reaction temperature, led to conditions that had little effect on DNA topology (12) but caused an inability to assemble DNA into nucleosomes. Here we report the possible reasons for the failure in earlier attempts (44, 52) to assemble physiological chromatin in transcriptionally active HeLa cell extracts (40) and describe the ionic strength, cofactor requirements, stoichiometry, kinetics, and mechanism of nucleosome assembly in a mammalian cell extract.

MATERIALS AND METHODS

Reagents, virus, and viral DNA. Nucleotides and nucleotide analogs were from Pharmacia; poly-L-glutamic acid (M_w 106,000) was from ICN. HeLa whole-cell extract, calf thymus topoisomerase 1, and restriction endonucleases were from Bethesda Research Laboratories; calf thymus histones and micrococcal nuclease were from Worthington Biochemicals. Two strains of SV40 were used, strain 776 (50) and strain VA-45-54 (58). Strain VA-45-54 differs from strain SV-S (11) principally by having an extra 21 base pairs (bp) in two long repeats, so that instead of being 72 bp, the repeats are 93 bp in length (7). CVI cells were infected with plaque-purified virus at a multiplicity of infection of 2 to 3 PFU/cell. At 60 to 72 h postinfection, viral DNA was extracted (28) and purified by ethidium bromide-CsCl den-

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sity gradient centrifugation. DNA concentrations were measured spectrophotometrically (A_{260}/A_{280} ratio was 1.8 to 1.9).

HeLa cell extracts. HeLa cell extracts were prepared from suspension cultures (3×10^6 to 6×10^6 cells per ml) (S3 type), following a procedure described by Sugden and Keller (57) as adapted by Manley et al. (40), with several modifications. Cells were washed twice with 100 volumes of 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.0)–140 mM NaCl–1 mM $MgCl_2$. In most of the experiments, the extracts were prepared with a 0.4 M ammonium sulfate extraction of cells. However, we subsequently used two simple changes in the procedure which increased the histone content of the extract considerably without compromising the transcription activity. The ammonium sulfate extraction was done at 0.73 M, and final dialysis of the extract was done against 400 to 500 volumes of buffer with one change for 4 h. The conductivity of the extract was within 10% of that of the dialysis buffer. A 1-μl amount of the extract was able to assemble 16 to 25 ng of SV40 DNA. To prepare Mg^{2+}-free extract, $MgCl_2$ was omitted from every solution, including washing buffer. To determine the optimum ionic strength for assembly and transcription, several pilot extracts were prepared by reducing the KCl concentration by dialysis to 50 mM. In regular extracts, the Mg^{2+} concentration was 8 to 10 mM. Glu-extract was prepared by dialyzing the extract against 150 mM potassium glutamate (Sigma Chemical Co.). The protein concentrations of the extracts were between 12 and 20 mg/ml, as determined by the method of Bradford (9).

Covalently closed relaxed DNA. Supercoiled SV40 DNA was relaxed by adding 2 U of calf thymus topoisomerase I per μg of DNA in 20 mM Tris chloride (pH 8.0)–150 mM NaCl at 37°C for 1 h. To generate γ-^{32}P-labeled, covalently closed, relaxed circles, SV40 DNA was cleaved at the unique *Ban*I site, dephosphorylated, and end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) by using polynucleotide kinase (43). Subsequently, ligation was done in 25 to 30 ml (at a DNA concentration of 0.5 to 1 μg/ml) of a solution containing 25 mM Tris chloride (pH 7.6), 10 mM $MgCl_2$, 4 mM mercaptoethanol, 400 μM ATP, and 5 Weiss units of T4 DNA ligase (Boehringer Mannheim) per ml at 0°C for 12 to 16 h. The volume was reduced to 500 to 800 μl by repeated butanol extraction. The DNA was phenol-chloroform extracted, ethanol precipitated, and then separated by 1% agarose gel electrophoresis in the presence of 1 μg of ethidium bromide per ml for 18 to 20 h. Under these conditions, covalently closed supercoiled and relaxed circular DNA nearly comigrate. The DNA was electroeluted from the excised band of the gel, extracted three to four times with butanol, and concentrated by passage through an Elutip-D column (Schleicher & Schuell), followed by ethanol precipitation.

Nucleosome assembly assays. Assembly studies were carried out in Mg^{2+}-free or Mg^{2+}-containing (8 to 10 mM) extracts with 150 mM KCl (Cl-extracts) or 150 mM potassium glutamate (Glu-extracts). Incubations were done at 30 or 37°C for 60 or 30 min, respectively, in 50 to 100 μl of a solution containing 250 to 600 μg of extract proteins, 50 to 250 ng (2.5 to 15 μg/ml) of covalently closed relaxed SV40 DNA and 150 mM KCl or potassium glutamate in 10 to 15 mM HEPES–chloride, pH 7.9. This basic protocol was altered for particular experiments as described in the figure legends. Following assembly, the reactions were stopped on ice by adding 30 mM EDTA and digested with 100 μg of DNase-free RNase A (Sigma) per ml for 15 min at 37°C and then with 1% sodium dodecyl sulfate (SDS) and 100 μg of proteinase K (Boehringer Mannheim) per ml for 2 to 3 h at 37°C. Phenol-chloroform-extracted and ethanol-precipitated DNA was suspended in TAE buffer (39) and separated by electrophoresis in a 1 to 1.2% agarose gel containing TAE for 16 to 20 h. Gels were stained for 5 min in electrophoresis buffer containing 1 μg of ethidium bromide per ml and then exposed to 310-nm light for 1 min. This process was repeated two or three times. Following another 1 h of staining, gels were destained in deionized water for 8 to 10 h and photographed under UV illumination. When radioactively labeled DNA was assembled, RNase treatment was omitted, and gels were dried and autoradiographed by exposure to XAR-5 film at −70°C. Each extract was titrated to determine the optimum DNA concentration for assembly. For Glu-extracts, DNA titrations were usually done in the presence of 25 to 40 mM EDTA. Time course experiments showed that assembly was completed in 30 or 60 min at 37 or 30°C, respectively. Micrococcal nuclease digestion patterns of the chromatin assembled at 30 or 37°C were identical.

For micrococcal nuclease digestion of assembled chromatin, reactions were scaled up to 180 to 350 μl and either radiolabeled or unlabeled covalently closed relaxed SV40 DNA was assembled at 150 mM KCl or potassium glutamate in the presence or absence of cofactors and poly-L-glutamic acid as indicated in the figure legends. Following assembly at 37°C for 1 h, a sample was taken and held in ice. In the rest of the reaction mixes, $CaCl_2$ was adjusted to 3 mM and 40 U of micrococcal nuclease was added per 100 μl of reaction mix. Digestion was done at 30 or 37°C, and samples were taken at different time points. Digestion was stopped by adding 20 mM each EGTA (ethylene glycol tetraacetic acid) and EDTA, and the reactions were processed as described above. The digested DNA was separated by 1.5% agarose gel electrophoresis in TBE (39). The gels were photographed under UV illumination or autoradiographed.

RESULTS

Chromatin assembly in a HeLa cell extract. Nucleosome assembly was studied in HeLa whole-cell extracts. The extracts were dialyzed in a buffer with KCl (Cl-extract) or potassium glutamate (Glu-extract) in the presence or absence of exogenous Mg^{2+}. Potassium glutamate was included in these studies because at the K^+ ion concentration necessary for optimum assembly (150 to 200 mM; see below), Glu-extract supported pol II-mediated transcription, whereas Cl-extract did not (unpublished data). The extracts dialyzed in Mg^{2+}-free buffers still contained a certain amount of endogenous Mg^{2+}. This endogenous Mg^{2+} concentration was much lower in the Cl-extract than in the Glu-extract (see below). KCl is often used to study nucleosome assembly and transcription in vitro (33, 65). We initially used KCl because K^+ is the most abundant intracellular monovalent cation (3).

Covalently closed relaxed SV40 DNA was used as a substrate to study nucleosome assembly in vitro. Since each nucleosome assembled introduces one positive superhelical turn during assembly, deproteinization of the assembled chromatin will generate negatively supercoiled DNA (23, 32). Thus, the extent of assembly can be measured by determining the average number of superhelical turns following deproteinization of DNA and agarose gel electrophoresis (32). The limited histone content of a HeLa whole-cell extract allows the assembly of only a small amount of DNA, which is difficult to analyze by gel electrophoresis and ethidium bromide staining. This problem can be overcome

either by increasing the volume of the reaction mix or by adding trace amounts of radiolabeled covalently closed relaxed SV40 DNA in a small-scale reaction mix (see Materials and Methods). Some of the experiments described below employed the latter procedure.

Effect of Mg^{2+}, other cations, and polyanions on assembly in the absence of ATP. Nucleosome assembly in *Drosophila* extracts can occur in the absence of Mg^{2+} and ATP (45). Similarly, in prior studies these cofactors appeared to have no role in nucleosome assembly in HeLa extracts but caused catenation at higher DNA concentrations (52). However, the independent effect of these cofactors on nucleosome assembly had not been systematically investigated. In order to do this, we first asked what role Mg^{2+} alone plays in assembling nucleosomes in a HeLa extract.

Covalently closed relaxed or supercoiled SV40 DNA at the indicated concentrations (Fig. 1a) was added to a HeLa extract under standard transcription conditions (40) except that ATP was omitted and excess EDTA was added to some reaction mixes to chelate Mg^{2+}. Surprisingly, removal of Mg^{2+} was found to increase the assembly of nucleosomes at low DNA concentrations (compare lanes 4 and 5 with 1 and 2 or lanes 10 and 11 with 7 and 8). In the presence of EDTA, progressive increases in DNA concentrations gave proportionally less nucleosome assembly (compare lanes 1 to 3 with 4 to 6 or lanes 7 to 9 with 10 to 12), suggesting titration of histones, as described previously (36, 45, 52). Thus, different patterns of nucleosome assembly were observed in the presence and absence of Mg^{2+}. As expected, supercoiled DNA assembled better than the relaxed form, presumably because its assembly is energetically favored (23, 61).

The experiment shown in Fig. 1a was done at 60 mM KCl, which is the optimum ionic strength for in vitro transcription (40). However, we found that optimum nucleosome assembly in a HeLa extract occurred at 125 to 200 mM KCl or potassium glutamate (see below), which is close to physiological ionic conditions (3). Under these conditions, the Mg^{2+} interference was more evident in the Glu-extract (Fig. 1b).

Additional experiments were performed to verify that incomplete assembly in the presence of Mg^{2+} in a Glu-extract was Mg^{2+} specific. In one case, the Mg^{2+} concentration was increased from 3 to 10 mM; in another, Mg^{2+} was progressively chelated by increasing EDTA concentrations. In the absence of EDTA, 3 mM Mg^{2+} was sufficient to inhibit assembly significantly, and higher concentrations had no further effect (Fig. 1c). Unexpectedly, at 10 mM Mg^{2+}, substoichiometric or stoichiometric amounts of EDTA enhanced the inhibition of assembly (Fig. 1c, compare lane 9 with 10 and lane 4 with 5 and 6). Still higher EDTA concentrations restored efficient nucleosome assembly (lanes 11 and 12). This indicates that inhibition of nucleosome assembly depends upon a critical free Mg^{2+} concentration. When stoichiometric amounts of Mg^{2+} and EDTA were present during assembly, some intermediate DNA bands were generated following deproteinization (Fig. 1c, lane 10). These intermediate bands may represent incomplete winding of DNA around nucleosomes or unusual secondary structures of DNA that were generated under this assembly condition.

As a further confirmation of the Mg^{2+} interference in assembly, we used Mg^{2+}-free Cl-extracts in which, compared with a similar Glu-extract, the endogenous Mg^{2+} concentration was not high enough to interfere significantly with assembly. In these Cl-extracts, as little as 500 μM Mg^{2+} almost totally inhibited assembly (Fig. 1d, compare lane 2 with lane 6). Indeed, even lower levels of endogenous Mg^{2+} were sufficient to prevent complete assembly (compare lane 2 with lanes 8 and 9). Since EGTA could not substitute for EDTA in preventing Mg^{2+} interference (data not shown), we conclude that the effect was not due to the presence of a chelator alone.

To test whether other cations could mimic the Mg^{2+} effect, nucleosomes were assembled in the presence of several di-, tri-, and polyvalent cations. Mn^{2+} was a more effective inhibitor than Mg^{2+}; Fe^{3+} was a less effective inhibitor than Mg^{2+}, but a little more effective than spermidine; Cu^{2+} did not inhibit assembly (Fig. 1e). Ca^{2+} was as effective an inhibitor as Mg^{2+} (data not shown).

The observation that cations in general inhibited nucleosome assembly led us to investigate the role of polyanions on nucleosome formation. Previously, acidic proteins such as nucleoplasmin (35), poly-L-glutamic acid (54), and high-molecular-weight RNAs (46) have been shown to facilitate nucleosome assembly in vitro. We asked whether Mg^{2+}-mediated inhibition of nucleosome assembly could be relieved by poly-L-glutamic acid. Relaxed SV40 DNA was assembled in an Mg^{2+}-free Cl-extract in the presence of 5 mM Mg^{2+} and different concentrations of poly-L-glutamic acid. Poly-L-glutamic acid at 125 μg/ml completely overcame the presence of 5 mM Mg^{2+} and allowed efficient assembly (Fig. 1f). At poly-L-glutamic acid concentrations below 125 μg/ml, assembly was less efficient, as evidenced by the observation that part of the relaxed DNA migrated as topoisomers above the supercoiled marker (M2). We conclude that polyglutamic acid, like EDTA, relieves Mg^{2+}-mediated interference with nucleosome assembly.

Assembly occurs at physiological ionic strength. The ionic conditions used in previous studies of in vitro assembly were 90 to 100 mM NaCl (35, 45), 80 mM NH_4^+ (52), and 90 mM KCl (25). We explored the effect of ionic strength on nucleosome assembly, with the eventual goal of correlating assembly and transcriptional activity. Covalently closed relaxed SV40 DNA was assembled at different ionic strengths in a Cl-extract. In this extract, the highest salt concentration tested produced maximum assembly in the absence of Mg^{2+}, and ATP-dependent assembly (see below) was found to have similar salt dependence (Fig. 2b and c). In Glu-extract, when Mg^{2+} was chelated by relatively high EDTA concentrations (40 mM), maximum assembly was attained at 150 mM K^+ (Fig. 2d, lane 1). Interestingly, the Mg^{2+}-mediated interference with assembly was relieved by raising the K^+ concentration to 500 mM (compare lane 2 with 3). This shows that Mg^{2+} interference can be overcome by increasing the K^+ concentration to a nonphysiological level. The lack of assembly (lane 5) at a still higher salt concentration (1 M) may have been due to inactivity of topoisomerase I.

Assembly depends on DNA concentration. Optimum in vitro nucleosome assembly has been observed to occur when DNA and purified core histones are mixed at a DNA-to-histone ratio (wt/wt) of 1:1 to 1:2 (23) or 1:0.9 (24), a histone-DNA stoichiometry roughly similar to that of native chromatin. The endogenous histones of the HeLa extract could assemble a small amount of DNA. Thus, we asked whether, under conditions in which assembly was only partial, it could be enhanced by adding exogenous calf thymus histones. Nucleosome assembly was inhibited when histones were added in large excess (Fig. 3a, compare lane 1 with 4), whereas nucleosome formation was promoted when the exogenous histone-to-DNA ratio was approximately 1:1 (compare lane 3 with 6).

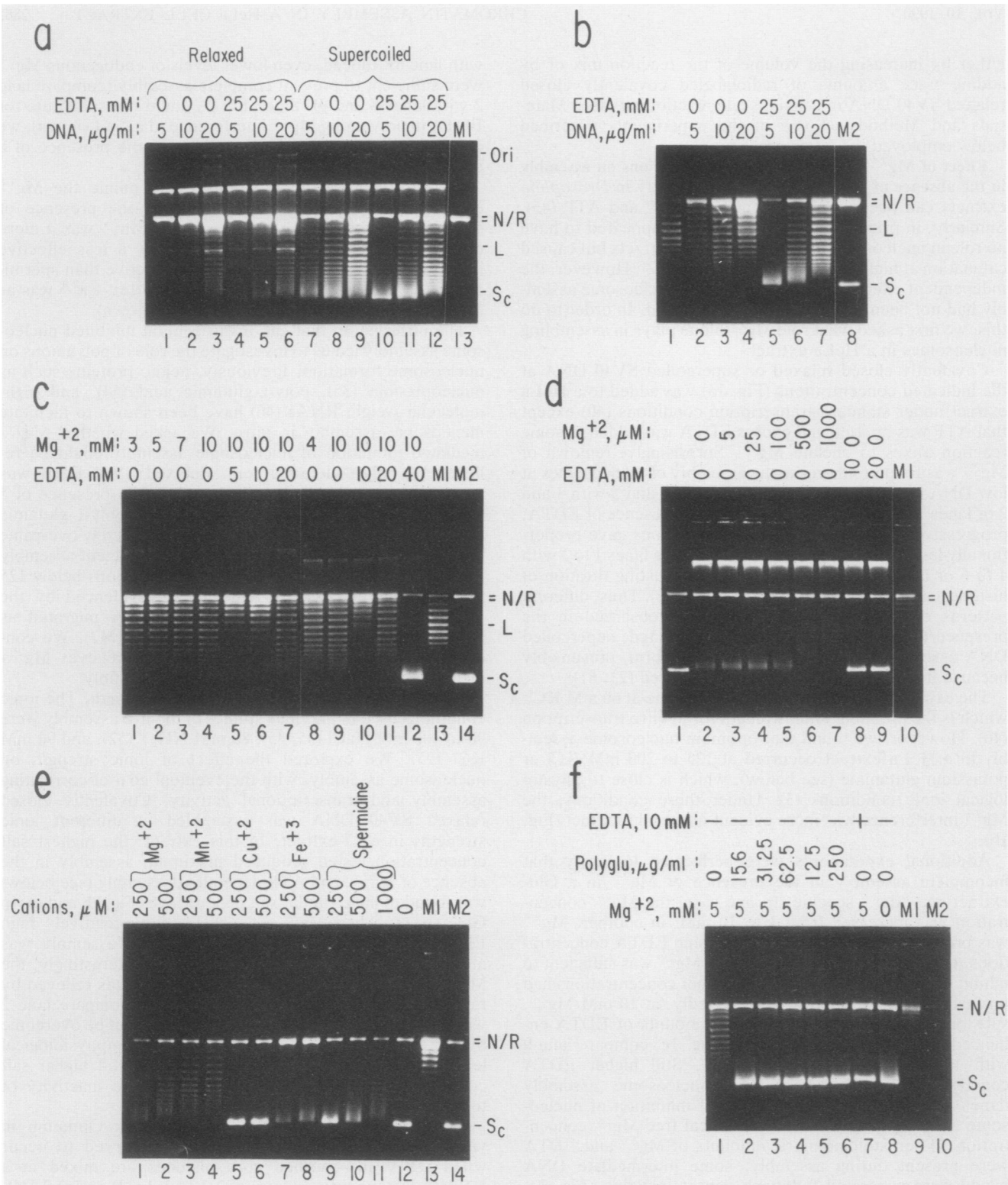


FIG. 1. Mg^{2+} and other cations inhibit and anions facilitate nucleosome assembly in HeLa extracts. Following assembly, the deproteinized DNA was separated by electrophoresis in 1.2% agarose gels. N/R, Nicked or covalently closed relaxed SV40 DNA; S_c, supercoiled SV40 DNA; ori, origin of the gel. Lanes M1 and M2 contain untreated relaxed and supercoiled DNA, respectively, in this and all subsequent figures. (a) Assembly of relaxed or supercoiled SV40 DNA was carried out in KCl (Cl-extract, obtained from Bethesda Research Laboratories) under standard transcription conditions at 7 mM Mg^{2+} (40) except that ATP was omitted or EDTA was added as indicated. (b) Assembly of relaxed SV40 DNA at 150 mM potassium glutamate (Glu-extract) containing 5 mM Mg^{2+} and EDTA as indicated. Lane 1 is untreated relaxed DNA. (c) Mg^{2+} inhibition and EDTA stimulation of assembly of 4 μg of covalently closed relaxed SV40 DNA per ml. Lanes 1 to 7 and lanes 8 to 14 are results from two different experiments carried out in Mg^{2+}-free and Mg^{2+}-containing Glu-extracts, respectively. (d) Mg^{2+} interference with assembly in an Mg^{2+}-free extract at 150 mM KCl. Note that this extract contains some endogenous Mg^{2+} that could be chelated by EDTA. (e) Nucleosome assembly in an Mg^{2+}-free Cl-extract in the presence of $MgCl_2$, $MnCl_2$, $CuCl_2$, $FeCl_3$, or spermidine hydrochloride. (f) Nucleosome assembly in a Cl-extract in the presence of 5 mM Mg^{2+} and different concentrations of poly-L-glutamic acid (Polyglu).

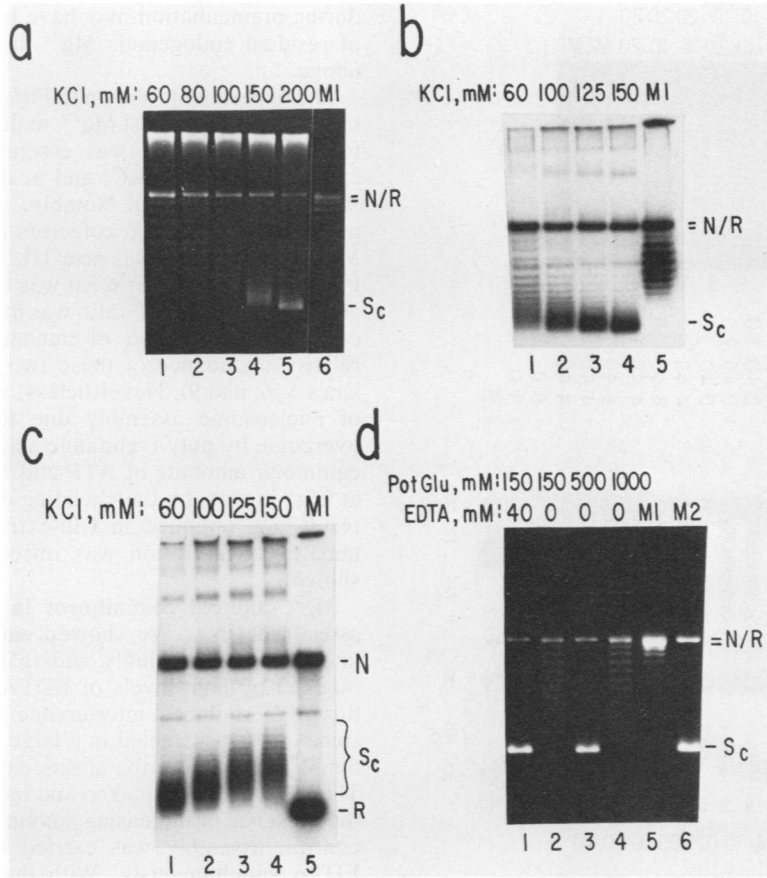


FIG. 2. Nucleosome assembly depends on ionic strength, and a high salt concentration relieves Mg^{2+} interference. (a) SV40 DNA (2.5 μg/ml) was assembled at 60 to 200 mM KCl in an Mg^{2+}-free extract. (b and c) γ-^{32}P-labeled (0.05 μg/ml) plus covalently closed relaxed SV40 DNA (2.5 μg/ml) assembled at 60 to 150 mM KCl in 3 mM Mg^{2+} and 3 mM ATP at 30°C for 60 min. (d) Relaxed SV40 DNA (2.5 μg/ml) was assembled at different concentrations of potassium glutamate (PotGlu) in 5 mM Mg^{2+}. Samples were analyzed as described in the legend to Fig. 1 except for panel c, where electrophoresis was done in the presence of 30 μM chloroquine. Gels of ^{32}P-labeled samples were dried and autoradiographed.

The inhibitory effect of excess histones became more evident when the endogenous histones of the extract were presented with lower DNA concentrations. About 0.05 μg of γ-^{32}P-labeled, covalently closed relaxed SV40 DNA per ml together with various amounts of unlabeled relaxed DNA were added to an Mg^{2+}-free or Mg^{2+}-containing Glu-extract in the presence of EDTA. After subsequent deproteinization, the DNA was analyzed by electrophoresis in the presence and absence of chloroquine. Maximum assembly in the two extracts tested occurred at the highest total amount of DNA tested, about 3 μg/ml (Fig. 3b and c, lanes 6 and 12). Almost no assembly was observed when the total DNA was about 60-fold less (lanes 1 and 7). Partial assembly was detected only when the total DNA concentration was raised to 1 μg/ml or more. A similar requirement for a low histone-to-DNA ratio was evident in ATP- and Mg^{2+}-dependent assembly (Fig. 3d). We conclude that some critical ratio of histone to DNA is apparently necessary for maximum nucleosome assembly in HeLa cell extracts.

Assembly in the presence of Mg^{2+} is ATP dependent. Nucleosome assembly in a HeLa cell extract was efficient in the absence of Mg^{2+} and was inhibited by Mg^{2+}. The Mg^{2+} interference was enhanced at low EDTA concentrations and relieved at high EDTA concentrations. This suggested that it would be interesting to examine the effect of natural Mg^{2+}-binding ligands on the assembly process, molecules such as ATP and GTP. There is a potential problem in distinguishing the chelating and cofactor functions of ATP and GTP, because the cofactor activities usually require binding of Mg^{2+} or other divalent metal ions. However, cofactor activity can be separated from simple binding or chelating activity by examining other nucleoside triphosphates.

We studied nucleosome assembly in the presence of 4 mM Mg^{2+} at different concentrations of ATP (Fig. 4a and b). This ATP titration showed two distinct peaks of assembly, a slight one at 2.5 mM and a more substantial one at 20 mM (lanes 5 and 7). We suspected that assembly at low ATP-Mg^{2+} ratios was energy dependent while the assembly at high ATP-Mg^{2+} ratios was the effect of sequestering of Mg^{2+} by ATP. This interpretation was supported by the similarity of the effect of 10 or 20 mM ATP or GTP with that of EDTA (compare Fig. 1c with Fig. 4). The superhelical density of DNA assembled in the presence of EDTA was a little more than that in 20 mM ATP (compare lane 9 with 7 in Fig. 4a and b), suggesting greater EDTA chelation of Mg^{2+}. However, we found that while 20 mM ATP or GTP facilitated assembly, CTP could not (Fig. 4c). This shows that Mg^{2+} binding is not the only effect of the high ATP and GTP concentrations. Furthermore, the nonhydrolyzable ATP analog 5′-AMP-PNP, which can bind Mg^{2+} but fails to serve as an energy source, had no effect on assembly. This suggests that

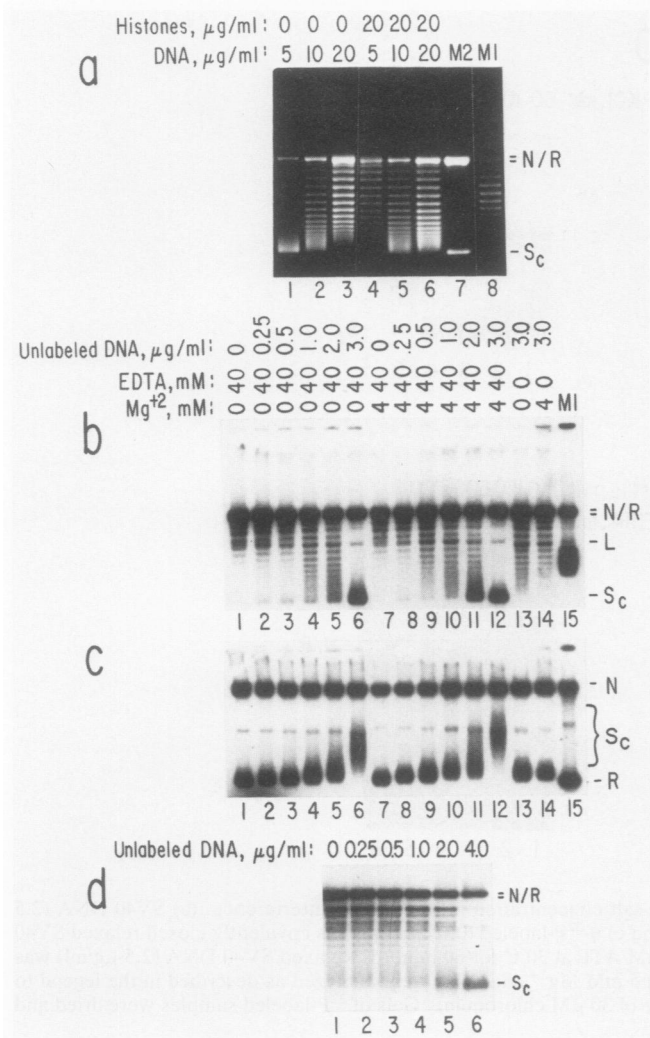


FIG. 3. Stoichiometric histone-to-DNA ratio promotes optimum nucleosome assembly. (a) Relaxed SV40 DNA assembled in the presence or absence of calf thymus histones (20 μg/ml) in 4 mM Mg^{2+}, 25 mM EDTA, and 150 mM potassium glutamate. (b and c) Titration of histones in an Mg^{2+}-free or Mg^{2+}-containing (final concentration, 4 mM) Glu-extract in EDTA as indicated with increasing amounts of unlabeled relaxed but a constant amount, 0.05 μg/ml, of γ-^{32}P-labeled relaxed SV40 DNA. (d) Similar to panel b except that DNA was assembled in 3 mM Mg^{2+} and 3 mM ATP. Samples were analyzed as for Fig. 1 except for panel c, where electrophoresis was done in the presence of 30 μM chloroquine.

the primary effect of low ATP concentrations on assembly in the presence of Mg^{2+} is the result of ATP hydrolysis.

The experiments described above suggest that some of the ATP was necessary to sequester free Mg^{2+} during assembly. We suspected that if Mg^{2+} served solely as a cofactor for ATP hydrolysis, then an assembly limited by low Mg^{2+} would be enhanced by its subsequent addition. Alternatively, if Mg^{2+} acted merely as an inhibitor, then its presence should at least prevent further assembly. To test this, DNA was incubated in the presence of different concentrations of ATP in an Mg^{2+}-free Glu-extract before more Mg^{2+} was added. Addition of Mg^{2+} following incubation of DNA in the presence of ATP facilitated assembly (Fig. 4d, compare lane 3 with lane 8), suggesting that ATP hydrolysis was necessary for the process. Increased assembly at 5 or 8 mM ATP during preincubation may have been the result of chelation of residual endogenous Mg^{2+} in the extract, as described above.

The experiments shown in Fig. 4 suggested that we should titrate the ATP against Mg^{2+} to determine the optimum ratio for assembly. DNA was assembled in an Mg^{2+}-free Cl-extract at 150 mM KCl and at different ratios of Mg^{2+} to ATP (Fig. 5a and b). Notably, maximum assembly in the presence of these two cofactors usually occurred when the Mg^{2+}-to-ATP ratio was near 1:1. However, the most revealing result of this experiment was that when ATP was present and the Mg^{2+}-to-ATP ratio was more than 1:1, DNA became catenated. The degree of catenation was controlled by the relative abundance of these two cofactors (Fig. 5a and b, lanes 5, 6, and 9). Nevertheless, the catenation or inhibition of nucleosome assembly due to excess Mg^{2+} could be overcome by poly-L-glutamic acid (Fig. 5c), suggesting that equimolar amounts of ATP and Mg^{2+} facilitated assembly, at least in part, by the chelating of Mg^{2+} by ATP. A similar result was obtained in Glu-extracts when the poly-L-glutamate concentration was raised to 500 μg/ml (data not shown).

Mg^{2+}-induced commitment in assembly and stability of assembled DNA. We showed earlier that Mg^{2+} alone can interfere with assembly and that this interference can be relieved by high levels of EDTA. Now we asked whether this Mg^{2+}-induced interference was irreversible. Nucleosomes were assembled in a large volume in the presence of 6 mM Mg^{2+} and in the absence of ATP for 30 min at 37°C. Then, samples were taken and incubated for 60 min more in the presence of increasing amounts of EDTA (Fig. 6a). As a control, assembly was carried out by adding Mg^{2+} and EDTA simultaneously. With the two-step reactions, very little assembly occurred even with the addition of 60 mM EDTA. This indicates that whatever inhibited state is reached in the presence of Mg^{2+} cannot easily be reversed to allow nucleosomes to form. A similar result was obtained when ATP was added instead of EDTA (data not shown). In contrast, when DNA was assembled at a low Mg^{2+}-to-ATP ratio, subsequent addition of Mg^{2+} stimulated further assembly (Fig. 4d). Next we asked whether the assembled DNA could be disassembled by a subsequent challenge with Mg^{2+}. The DNA was assembled in an Mg^{2+}-free Cl-extract in the absence of ATP or in a Glu-extract in the presence of EDTA and was subsequently challenged with different amounts of Mg^{2+} (Fig. 6b). No significant disassembly of nucleosomes was observed.

Assembled chromatin displays regularly spaced nucleosomes. Chromatin reconstituted from purified core histones usually has a random distribution of nucleosomes along the DNA (24, 34). A similar result was obtained when SV40 DNA was assembled in a HeLa cell extract under transcription conditions (52). However, assembly of DNA in a *Drosophila* extract in the absence of cofactors (45) or in a *Xenopus* oocyte extract in the presence of ATP and Mg^{2+} (25) showed a 200-bp regular spacing of nucleosomes. To examine nucleosome spacing under our conditions, chromatin assembled in the presence or absence of ATP and Mg^{2+} in a Cl-extract was partially digested with micrococcal nuclease. We saw a weak 200-bp periodic spacing of nucleosomes on DNA assembled in both cases (Fig. 7a and b). A similar nucleosome spacing was observed when CVI monkey cell nuclei were treated with methidium propyl-EDTA-Fe(II) (8) or HeLa cell nuclei were digested with micrococcal nuclease (data not shown).

We failed in repeated attempts to obtain better nucleo-

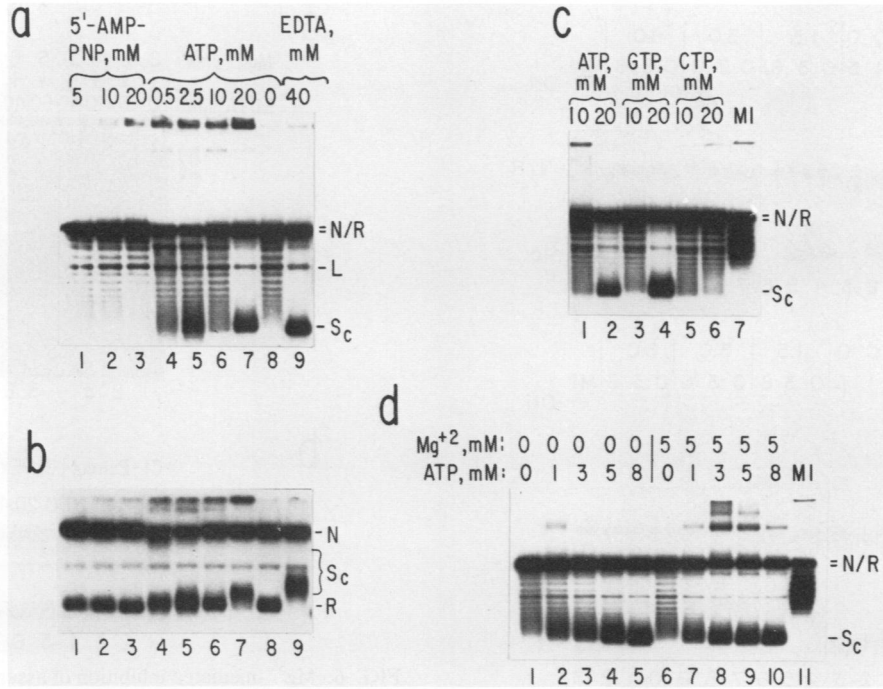


FIG. 4. Energy-dependent and -independent assembly in the presence of Mg^{2+}. Samples were analyzed as for Fig. 1 except in panel b, where electrophoresis was done in the presence of 30 μM chloroquine. (a and b) Unlabeled (3 μg/ml) plus γ-^{32}P-labeled (0.05 μg/ml) relaxed SV40 DNA was assembled at 150 mM potassium glutamate and 4 mM Mg^{2+}, at different concentrations of ATP or 5'-AMP-PNP (PNP). (c) Same as panel a except assembled in ATP, GTP, and CTP. (d) Two-step assembly; DNA concentrations were as in panel a in the presence of endogenous Mg^{2+} in Glu-extract with 0 to 8 mM ATP for 30 min at 37°C. Samples were taken at this point and reactions were stopped on ice with EDTA, SDS, and proteinase K. To the rest of the reaction mixes, Mg^{2+} was added up to 5 mM, and samples were incubated for another 30 min.

some ladders under the conditions described above. We suspected that this was because our Mg^{2+}-free extracts still had some endogenous Mg^{2+}. If this were the case, the results described above suggested that the Mg^{2+}-mediated interference with nucleosome assembly might be overcome by poly-L-glutamic acid. Several reports (54, 55) showed that poly-L-glutamic acid at concentrations up to 2 mg/ml did not interfere with micrococcal nuclease activity in vitro. In an attempt to overcome the putative Mg^{2+}-mediated interference with nucleosome assembly and also to rule out the topological requirement for assembly, we assembled linear SV40 DNA in an Mg^{2+}-free Cl-extract containing 125 μg of poly-L-glutamate per ml in the presence or absence of 5 mM Mg^{2+}, 1 mM ATP, and 5 mM creatine phosphate. The results (Fig. 7d) suggested that removal of trace amounts of free Mg^{2+} did not enhance the production of regularly spaced nucleosomes. However, when ATP hydrolysis was allowed to occur in the presence of poly-L-glutamate, the stability of the chromatin was enhanced considerably, as judged by the clarity of the ladder of DNA fragments and quantitative micrococcal nuclease digestion into oligo- and mononucleosomes (Fig. 7c). Even clearer ladders were observed when 500 instead of 125 μg of poly-L-glutamate per ml was used at very low and high ATP concentrations (Fig. 7e and f). Increasing the poly-L-glutamate concentration up to 1 mg/ml in the absence of ATP and Mg^{2+} did not enhance stable chromatin formation (data not shown). We conclude that regularly spaced nucleosomes can assemble in HeLa cell extracts either in the absence of Mg^{2+} or in the presence of Mg^{2+} and ATP, but that ATP hydrolysis increases the stability of the chromatin considerably. The periodicities of nucleosomes as determined by polyacrylamide gel electrophoresis of micrococcal nuclease-digested chromatin, measured under nondenaturing and denaturing conditions, were 150 to 165 bp and 185 to 195 bp, respectively, depending on whether 5 mM Mg^{2+}, 1 mM ATP, and 5 mM creatine phosphate were absent or present (S. Banerjee, unpublished results).

DISCUSSION

Cation-specific inhibition of nucleosome assembly. We describe an in vitro system that supports nucleosome assembly in HeLa whole-cell extracts. In this system, several di-, tri-, and polyvalent cations inhibit assembly. Though it has been hinted that high concentrations of Mg^{2+} or ATP alone could inhibit nucleosome assembly in a *Xenopus* oocyte extract, such an effect was not observed previously in HeLa extract (44, 52) or in purified reconstitution systems (24). The inhibition of nucleosome assembly we saw was cation specific but did not strictly correlate with the size or the number of charges of the cations. For example, spermidine was not as effective as Mg^{2+}, and Mn^{2+} was more effective than Mg^{2+} in inhibiting nucleosome assembly. Two kinds of interaction of the cations with extract proteins or DNA are conceivable (41), electrostatic shielding and counterion binding. Since different divalent cations at the same ionic strength show different effects, counterion binding to the proteins or DNA is the most likely explanation for this inhibition.

How might this cation-specific inhibition occur? One possibility is inactivation of proteins in the extract that are

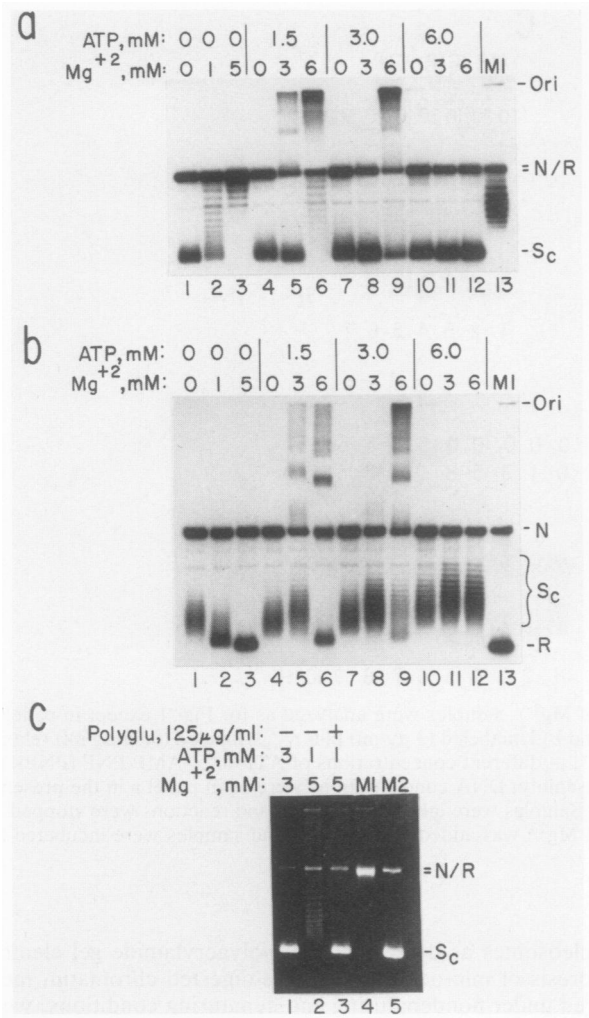


FIG. 5. Energy-dependent nucleosome assembly occurs at a critical ATP-to-Mg^{2+} ratio; poly-L-glutamic acid counteracts the inhibition caused by excess Mg^{2+}. (a and b) Unlabeled (2.5 μg/ml) plus γ-^{32}P-labeled (0.05 μg/ml) relaxed SV40 DNA assembled at 150 mM KCl at different ATP-to-Mg^{2+} ratios at 30°C for 60 min. (c) Covalently closed, relaxed, unlabeled SV40 DNA was assembled at a concentration of 15 μg/ml, in ATP, Mg^{2+}, and the presence or absence of 125 μg of poly-L-glutamic acid (Polyglu) per ml. Samples were analyzed as for Fig. 1 except for panel b, where electrophoresis was done in the presence of chloroquine.

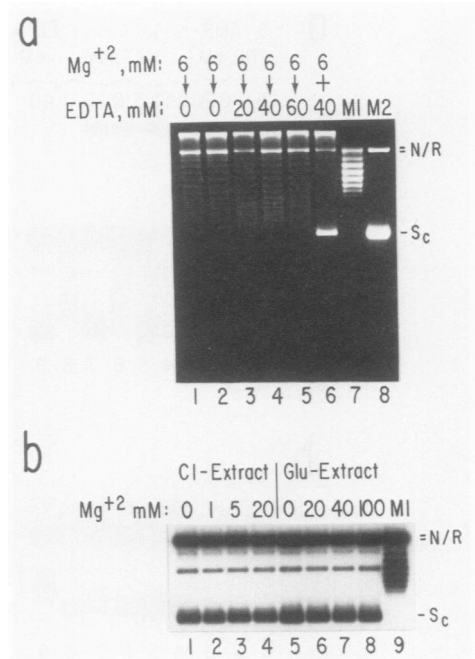
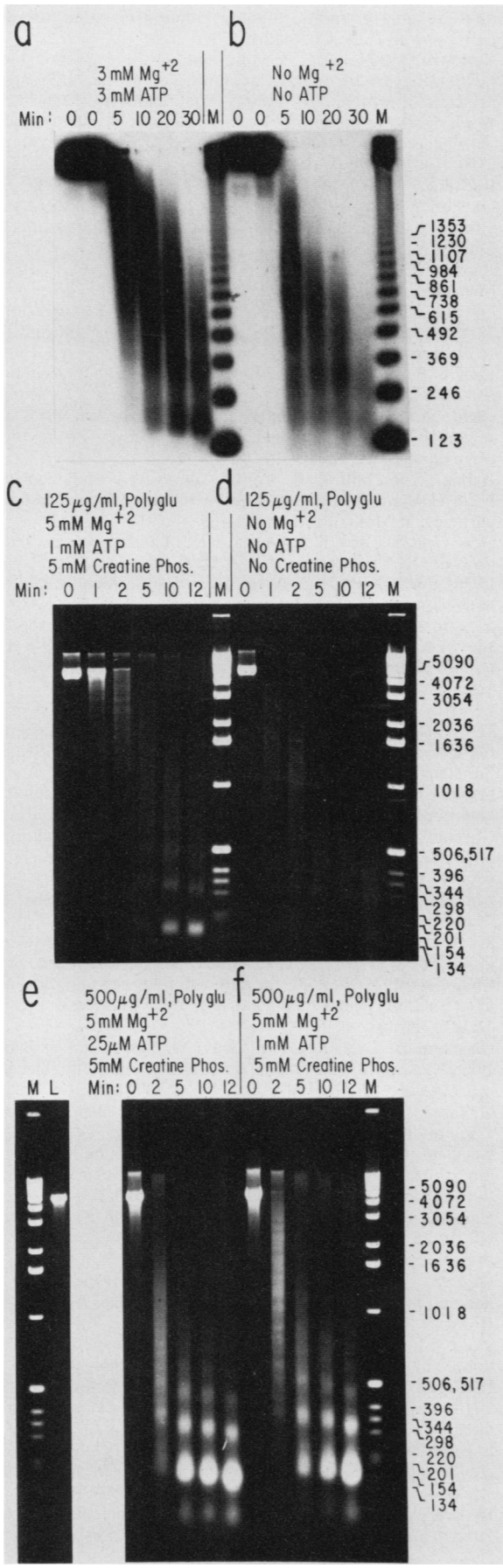


FIG. 6. Mg^{2+}-mediated inhibition of assembly is irreversible, but Mg^{2+} fails to cause disassembly of already assembled chromatin. (a) Covalently closed relaxed SV40 DNA (2.5 μg/ml) was assembled in a 300-μl reaction mix in a Glu-extract in the presence of 6 mM Mg^{2+} for 30 min at 37°C. Five samples (55 μl) were taken. Assembly in one sample was stopped immediately. To the rest, 0, 20, 40, or 60 mM EDTA was added, and the samples were further incubated for 60 min. As a control, 6 mM Mg^{2+} and 40 mM EDTA were added simultaneously (lane 6). Unlabeled (2.5 μg/ml) plus γ-^{32}P-labeled (0.05 μg/ml) relaxed SV40 DNA assembled in an Mg^{2+}-free Cl-extract or in a Glu-extract in the presence of 30 mM EDTA for 60 min at 30°C. Following assembly, different amounts of Mg^{2+} were added to the samples and incubation was continued for 60 min more.

essential for nucleosome assembly. Alternatively, cations might stimulate a protein in the extract in a way that blocks subsequent nucleosome assembly. Finally, cations may have a direct effect on the DNA. Recent studies suggest that divalent cations can induce and stabilize sequence-directed bending of roughly 200-bp sequences of kinetoplast minicircles or roughly 200 bp near the SV40 DNA replication terminus (36). Anomalous electrophoretic retardation of bent DNA is enhanced in the presence of 5 to 10 mM Mg^{2+} (17). Mg^{2+}-induced conformational alterations of SV40 DNA lead to nuclease sensitivity of several distinct sequences, including its control elements (31).

It is evident that DNA sequences can determine the position of nucleosomes (14, 51), and their local conformations might influence the extent of assembly (29, 30, 47, 48). The structural parameters of a standard B-form DNA in solution are influenced by the sequence of the DNA (10), temperature (16, 60), and the concentration and type of counterions in solution (27, 49, 63). To characterize the effect of Mg^{2+} on the global conformation of SV40 DNA, we relaxed supercoiled SV40 DNA with purified topoisomerase I in the presence or absence of Mg^{2+} and analyzed the resulting species by electrophoresis in Mg^{2+}-free buffer in the presence or absence of chloroquine. We found that 5 to 10 mM Mg^{2+} underwound SV40 DNA by the equivalent of two superhelical turns, an increment of winding angle of 0.137°/bp (data not shown). This supports our speculation that cation-mediated inhibition of nucleosome assembly could be caused by alterations in DNA structures. The observation that the Mg^{2+}-mediated inhibition of assembly could be relieved by a high K^+ concentration suggests that these two cations compete with each other to bind to the same target sites. Interestingly, K^+ at 100 to 200 mM concentrations has a minimal effect on the winding of duplex DNA (6).

Assembly depends on salt and DNA concentrations. Optimum nucleosome assembly in HeLa cell extracts occurs at physiological ionic conditions. Poor assembly at low salt concentrations may be caused by the inability to form normal nucleosome cores rather than by any interference in the binding of these cores. Both ionic bonds and hydrophobic interactions play important roles in generating histone octamers (15, 18). Nucleosomes undergo various structural transitions in response to the ionic strength of the environment (38, 66).



When histones were in large excess, assembly was severely inhibited. This observation is in agreement with previous reports (21, 55). Excess core particles may inhibit the assembly by nonspecific binding to the DNA or aggregation into inactive species.

Mg^{2+}- and ATP-dependent nucleosome assembly. Mg^{2+} alone inhibits nucleosome assembly. However, Mg^{2+} in the presence of at least equimolar amounts of ATP facilitates assembly by a pathway that requires ATP hydrolysis. Moreover, excess Mg^{2+} in the presence of ATP either inhibits assembly or promotes catenation of circular DNA. This inhibitory effect of excess Mg^{2+} can be circumvented by adding a sufficient amount of ATP, GTP, creatine phosphate, EDTA, or polyglutamic acid. These results suggest that at least a portion of the total amount of ATP was necessary to deplete free Mg^{2+} at the initial phase of assembly.

Energy-dependent nucleosome assembly of circular DNA in *Xenopus* oocyte extracts has been reported (25), but it is controversial (52, 54, 64). Our results suggest that nucleosomes can assemble in HeLa cell extracts in the absence of ATP and Mg^{2+}. However, in our system ATP hydrolysis substantially enhances the stability of the chromatin, possibly by phosphorylating histones and other nonhistone structural proteins. Histone phosphorylation has been correlated with the variable compactness and configuration of mitotic or metaphase chromatin (1, 2). Our results are consistent with a recent report in which stable histone-DNA interactions in *Xenopus* oocyte or egg extracts were shown to require ATP hydrolysis (5). Our finding that ATP hydrolysis is required for stable chromatin formation but is independent of DNA topology suggests that topoisomerase II is not required for generating physiologically spaced nucleosomes in HeLa extracts. These results also agree with those of Almouzni and Mechali (5).

The experimental system described here makes possible a variety of new investigations on the dynamics of chromatin structure and gene regulation. For instance, instead of reconstituting synthetic DNA (54) or tandemly repeated cloned DNA (53) into nucleosomes, large DNAs from any source can be assembled to mimic higher-order chromatin structure in this system. Numerous in vivo experiments suggest that nonhistone high-mobility-group proteins (26, 62), methylation of DNA (13, 59), and chemical modifications of histones, such as phosphorylation (2, 19, 22),

FIG. 7. Micrococcal nuclease digestion of assembled chromatin shows regular spacing of nucleosomes. Samples were analyzed as for Fig. 1 and 2 except that 1.5% agarose was used. Lane L, Untreated linear DNA; lane M, size standards (sizes indicated in base pairs). (a and b) Unlabeled (2.5 μg/ml) plus γ-^{32}P-labeled (0.28 μg/ml) relaxed SV40 DNA assembled in 180 μl of 150 mM KCl in the presence (a) or absence (b) of 3 mM Mg^{2+} and ATP at 37°C for 60 min. Samples (28 μl) were taken and assembly was stopped by adding SDS and proteinase K. To the rest of the reaction mixes, $CaCl_2$ was added to 3 mM, and chromatin was digested by adding 45 U of micrococcal nuclease at 30°C. (c and d) Linear SV40 DNA (4 μg) was assembled in a 400-μl reaction mix (containing 160 μl of Cl-extract) at 150 mM KCl and 125 μg of poly-L-glutamate (Polyglu) per ml in the presence (c) or absence (d) of 5 mM Mg^{2+}, 1 mM ATP, and 5 mM creatine phosphate (Phos.). (e and f) Same as panel d except 3 μg of DNA was assembled in the presence of 500 μg of poly-L-glutamate per ml at 25 μM (e) and 1 mM (f) ATP. Following assembly at 37°C for 60 min, a sample was taken, and the rest of the reaction mixes were digested with micrococcal nuclease (40 U/100 μl of reaction mix) at 37°C as indicated. All reactions were stopped by adding a mixture of EDTA and EGTA at final concentrations of 20 mM each and treated with RNase A and then with SDS and proteinase K (see Materials and Methods).

acetylation (4), and poly-ADP-ribosylation (20), have profound effects on chromatin organization and cell cycle-dependent gene expression. A somatic cell extract will facilitate study of these phenomena under controlled experimental conditions. It will also allow testing of whether positioning of nucleosomes influences the interaction of purified transcription factors with promoters or enhancers to modulate gene expression.

Conclusions. Our investigation of the role of various biochemical parameters on in vitro nucleosome assembly in a HeLa cell extract demonstrates that nucleosome assembly in this extract is more complex than was previously thought (44, 52). Ionic conditions, DNA and histone concentrations, cofactors, and possibly the structure of DNA play significant roles in nucleosome assembly in vitro. These results provide possible explanations for the inability of nonreplicating SV40 DNA to produce physiological chromatin in mammalian cell extracts (52, 56). Many questions remain unanswered. Though the spacing of the nucleosomes is physiological, we have no information on how this arises. We also do not know whether H1 participates in the nucleosome assembly, despite its presence in amounts equimolar to other histone species in the extract (52). In the *Xenopus* system, the role of an additional assembly factor, nucleoplasmin, was demonstrated (35). Whether such factors exist in a mammalian cell extract requires investigation. Finally, it remains to be determined whether the cation-mediated inhibition of nucleosome assembly described here is physiologically relevant.

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