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Binding of histones H1 and H5 and their globular domains to four-way junction DNA

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ABSTRACT **We have compared chicken erythrocyte linker histones H1 and H5 binding to a synthetic four-way DNA junction. Each histone binds to form a single complex, with an affinity which permits competition against a large excess of linear duplex DNA. The affinity of H5 is higher than that of H1. The globular domain from either protein will also bind strongly, but in this case multiple binding occurs. Binding of intact H1 is inhibited by cations: Mg^{2+} and spermidine are very effective, Na^{+} much less so. This inhibition is not likely to be a general ion-competition effect, for Mg^{2+} is much less effective in inhibiting the binding of H1 to linear DNA. Instead, the inhibition of binding may be due to ion-dependent changes in the conformation of the four-way junction, which are known to occur under similar conditions. These results strongly suggest that the angle formed between the arms of the DNA junction could be a major determinant in the interaction of H1 with DNA crossovers.**

Many studies have indicated that histone H1 and its variants interact with linker DNA in chromatin and are associated in particular with the entrance and exit of the DNA to and from the nucleosomal core particle (reviewed in refs. 1 and 2). Since many electron microscopy studies indicate that the DNA crosses itself upon entering and exiting the nucleosome (e.g., ref. 3), it would seem likely that DNA crossovers would provide preferential sites for H1 binding. Early evidence suggestive of this came from studies showing that H1 interacts preferentially with highly supercoiled DNA (4–6). However, the interpretation of these experiments was complicated by the fact that under high torsional stress DNA can also adopt a variety of non-B conformations, which might themselves provide preferred sites for H1 binding. In recent studies we have shown that H1 will exhibit a preference for even weakly supercoiled DNA, where such non-B structures are not formed, as compared with relaxed DNA (7). Others have suggested that four-way junction (4WJ) DNA should be structurally similar to DNA crossovers (8). 4WJ DNA is the core structure of cruciform DNA (which may extrude from palindromic sequences under torsional stress) and the Holiday junction (which forms during homologous recombination). Stable 4WJs can also be formed by annealing appropriately chosen oligonucleotides *in vitro* (9), and thus provide a convenient model system to study proteins which might interact specifically with this type of DNA structure.

These previous results and the demonstration by Bianchi *et al.* (10) that the nonhistone protein HMG1 binds preferentially to 4WJ DNA have led us to investigate the possibility that such structures might also be preferred sites for histone H1 binding. In a recent communication (11) we have shown that the same synthetic 4WJ used by Bianchi (12) binds to H1 strongly, even in the presence of an excess of nonspecific competitor linear DNA. This binding preference was not exhibited by linear DNAs containing the same sequences or by incomplete junctions. Thus, the structure of the 4WJ elicits a specific binding which is much stronger than the general binding of linker histones to linear DNA. To elucidate the significance of this finding it is obviously important to establish fully the conditions for binding of H1 to 4WJ DNA. This is of particular significance since others have shown that the ionic environment has a substantial effect on the conformation of the 4WJ itself (reviewed in ref. 13).

The linker histone H5 is specific for transcriptionally inactive erythrocytes of birds and fish (14, 15). It is similar in sequence and structure to H1 but has multiple lysine-to-arginine substitutions relative to H1. It is believed, but not known with certainty, that H5 occupies sites in chromatin that are similar to those taken by H1. Therefore, it seemed important to extend our studies to include this special lysine-rich histone.

All linker histones share a common three-dimensional structure—there exists a folded globular region which is flanked by less structured N- and C-terminal "tails" (16, 17). The tails contain a high concentration of charged amino acid residues. We felt it important to examine the roles of these different histone regions in the specific binding to 4WJ.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized with an Applied Biosystems 380B DNA synthesizer using phosphoramidite chemistry. The oligonucleotides were purified by denaturing polyacrylamide electrophoresis and reverse-phase chromatography on silica gels as described (18), except that SDS was omitted from the gel extraction buffer because it interfered with the enzymatic labeling of the oligonucleotides. Histones H1 and H5 were purified from chicken erythrocytes under nondenaturing conditions (19). The globular domains of H1 and H5 were prepared as described (7). The concentrations of H1 and H5 and their globular domains were determined spectrophotometrically by using extinction coefficients ($ml{\cdot}cm^{-1}{\cdot}mg^{-1}$, 230 nm) of 1.85 for H1 and H5 (20), 2.8 for the globular domain of H1, and 4.5 for the globular domain of H5 (21). We also determined the concentrations of H1 and H5 by amino acid analysis and derived values that were smaller by a factor of about 0.4 for both H1 and H5 than those derived by the spectroscopic methods. Salmon testis DNA was purchased from Sigma, sonicated to a size range of 100–2000 bp, and phenol/chloroform extracted and ethanol precipitated.

Construction of the 4WJ. The four oligonucleotides used to form the junction were of exactly the same sequences as those described by Bianchi (12). One oligonucleotide was radiolabeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The four oligonucleotides were then annealed in 10 mM

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Abbreviations: 4WJ, four-way junction; GH1 and GH5, globular domains of histones H1 and H5, respectively.
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Tris·HCl, pH 7.45/100 mM NaCl/1 mM EDTA by heating at 70°C for 3 min and cooling to room temperature over a period of 4–6 hr. The structure was then purified by polyacrylamide gel electrophoresis as described by Bianchi (12). The DNA was concentrated with a Centricon-10 microconcentrator (Amicon) and stored in 10 mM Tris·HCl, pH 7.45/100 mM NaCl/1 mM EDTA on ice. The formation of the 4WJ structure was verified by digestion with restriction endonucleases predicted to cut in specific arms only if duplex DNA had been formed (data not shown).

Analysis of Protein–4WJ DNA Interactions. Interaction of proteins with 4WJ DNA was studied by electrophoretic mobility-shift assays. The proteins were incubated with the indicated amounts of junction DNA in 20–40 μl of 10 mM Tris·HCl, pH 7.45/15.6 mM NaCl/5% (wt/vol) Ficoll 400. In some experiments, indicated amounts of $MgCl_2$, NaCl, and spermidine were included in this binding buffer. Sonicated salmon testis DNA, when used as competitor, was added at 50 μg/ml. Incubation was for 45 min at 20 ± 0.1°C. The samples were applied to 6.5% polyacrylamide gels (15 cm long and 1.5 mm thick) in 50 mM Tris base/50 mM glycine, pH 8.9, that had been extensively preelectrophoresed. Electrophoresis was at room temperature for 4–4½ hr at 100 V. The gel was then dried, and autoradiography was performed overnight at −80°C with intensifying screens.

Analysis of H1–Linear 146-bp DNA Interactions. Linear 146-bp DNA was purified from chicken erythrocyte core particles by repeated phenol/chloroform extractions. The binding of H1 to this mixed-sequence DNA was studied in 6% polyacrylamide gels run in the same manner as described above for the studies with 4WJ DNA. The indicated amounts of 146-bp DNA and H1 were incubated in the same binding buffer used in the studies with 4WJ DNA, either with or without an additional 10 mM Mg^{2+}. For these studies no competitor DNA was used, and the gels were stained with ethidium bromide.

RESULTS

Binding of Linker Histones H1 and H5 to 4WJ DNA. We studied the interaction of the linker histones H1 and H5 with 4WJ DNA by analyzing the protein–DNA complexes in DNA band-shift gel electrophoresis experiments. Fig. 1*A* shows a titration of H1 binding to 4WJ DNA in the absence of competitor DNA, and Fig. 1*B* shows the equivalent experiments using H5. Like H1, H5 formed a single specific complex with the 4WJ structure. The relative retardation of the band observed with H5 was, however, much smaller than that found with H1. Furthermore, the complex was detectable at about 5-fold lower H5 concentration than in the case of H1. Formation of insoluble aggregates that did not enter the gels was also observed at lower concentrations of H5 than of H1. In the presence of competitor DNA (Fig. 1 *C* and *D*), insoluble complexes did not form. Under these conditions, H5 again bound more avidly than H1 to the junction, since most 4WJ DNA was bound at about 15 nM H5, whereas at least ≈100 nM H1 was needed to achieve comparable levels of binding.

Binding of the Globular Domains of the Linker Histones to 4WJ DNA. Both H1 and H5 consist of a globular domain of about 80 amino acid residues with extended, basic N- and C-terminal tails (16, 17). The globular domains are trypsin-resistant and can be isolated after limited trypsin digestion of the histones. We studied the binding of these globular domains (GH1 and GH5) to the synthetic 4WJ DNA in the presence of sonicated salmon testis DNA as competitor. When GH1 was mixed with 4WJ DNA, binding was observed to occur in at least three stages (Fig. 2*A*): with increased GH1 concentration one bound DNA band was subsequently replaced by another one of lower mobility. Under exactly the same conditions, the intact H1 molecule produced only a single defined complex (see Fig. 1). Experiments with GH5 produced essentially the same results as those with GH1 (Fig. 2*B*). However, in contrast with the behavior of the intact histones, the retardation of the bands produced by GH5 was at least as great, if not greater, than that produced by GH1.

Ionic Effects on H1 Binding to 4WJ DNA. Some cations have been shown to have dramatic effects upon the structure of 4WJ DNA. It is thought that 4WJ DNA can exist as one of two different conformations, both of which are dependent upon the solution conditions. In the absence of specific cations a square planar configuration is observed (13). However, in the presence of sufficient concentrations of specific cations the junction folds into an X-shaped structure with two quasicontinuous, coaxially stacked helices (13). An interesting consequence of this bimorphism is that the angles between the four arms of the 4WJ vary

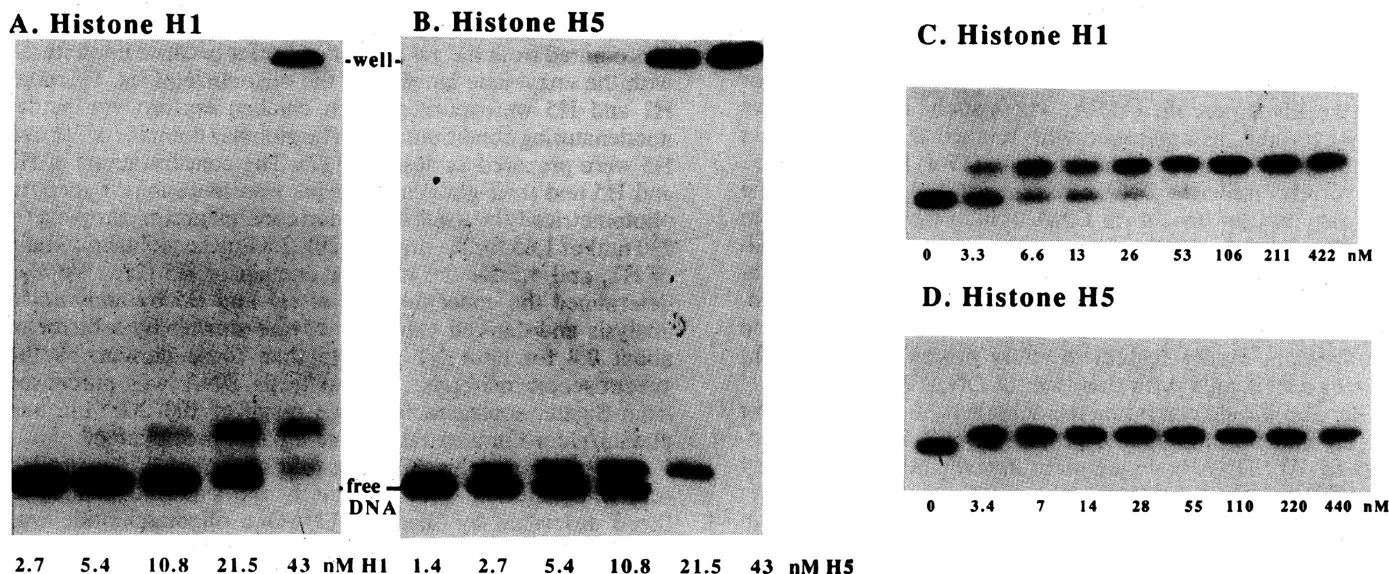
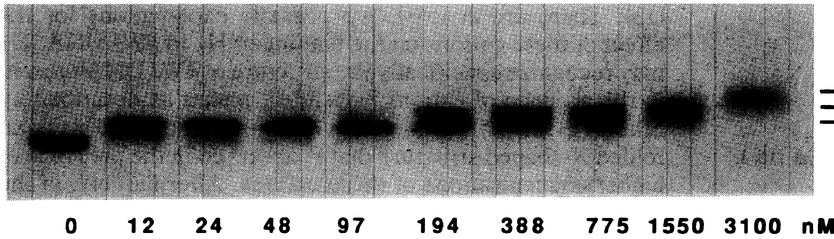


FIG. 1. Titration of binding of linker histones H1 and H5 to 4WJ DNA in the absence or presence of competitor DNA. Indicated concentrations of H1 (*A* and *C*) or H5 (*B* and *D*) were incubated with 4WJ DNA without competitor DNA (*A* and *B*) or with competitor DNA at 50 μg/ml (*C* and *D*). 4WJ DNA concentration was 4 nM in *A* and *B* and 2.1 nM in *C* and *D*. Electrophoretic DNA-band mobility-shift assays were performed as described in *Materials and Methods*. Polyacrylamide gel concentrations were 5%.

A. GH1



B. GH5

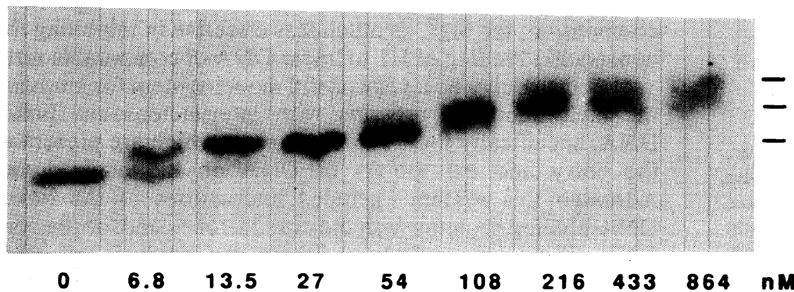


FIG. 2. Binding of GH1 and GH5 to 4WJ DNA. (*A*) Indicated concentrations of GH1 were incubated with 4.3 nM 4WJ DNA in 22 μl of 10 mM Tris·HCl, pH 7.45/15.9 mM NaCl/5.2% Ficoll containing sonicated salmon testis DNA at 50 μg/ml. An electrophoretic mobility-shift assay was performed with a 5% polyacrylamide gel concentration. (*B*) Indicated concentrations of GH5 were incubated with 2.1 nM 4WJ DNA in 20 μl of solution with sonicated salmon testis DNA as in *A*. The electrophoretic mobility-shift assay used a gel consisting of three different polyacrylamide concentrations in an effort to achieve better separation of the bands: top, 7.5% (2.5 cm deep); middle, 5% (3.5 cm deep); bottom, 3.5% (9 cm deep).

considerably between these two distinct conformations. Since H1 is presumably binding to two arms of the structure at once (see later discussion), the angle formed between the arms may be a key determinant in this interaction. Therefore, we have analyzed the effects of some specific ions on the binding of H1 to the 4WJ. Fig. 3*A* shows the effect of increasing levels of Mg^{2+}

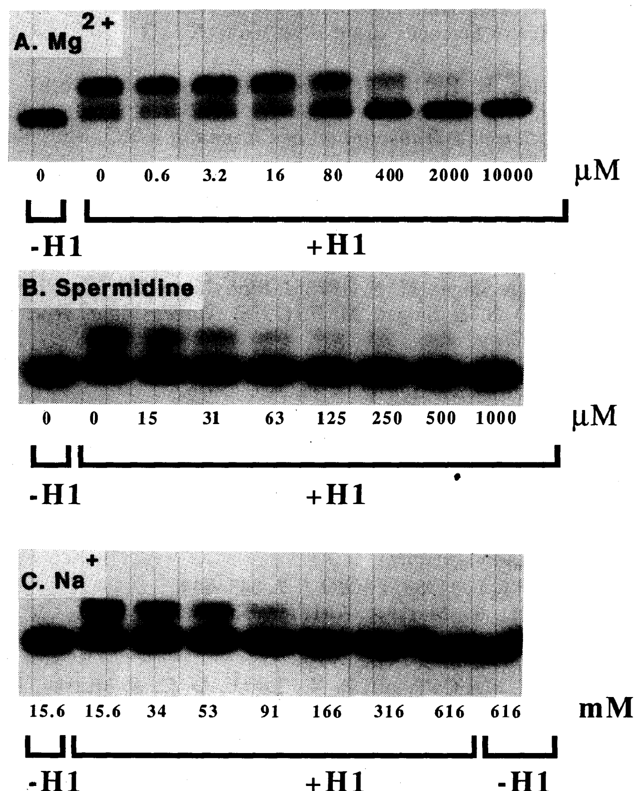


FIG. 3. Effects of Mg^{2+} (*A*), spermidine (*B*), and Na^{+} (*C*) on H1 binding to 4WJ DNA in the presence of competitor DNA. Concentrations were 52 nM H1 and 2.3 nM 4WJ (*A*) or 43 nM H1 and 5 nM 4WJ (*B* and *C*). Sonicated salmon testis DNA as competitor was at 50 μg/ml. The total concentrations of cations in the binding mixtures are indicated. Electrophoretic mobility-shift assays were performed as described.

on H1 binding in the presence of competitor DNA. Increasing the level of Mg^{2+} gradually decreased binding, with a titration midpoint at about 80 μM. Very similar results were obtained in the absence of competitor DNA (data not shown). The trivalent cation spermidine also interfered with the interaction of H1 and 4WJ DNA (Fig. 3*B*). In the presence of competitor DNA, spermidine concentrations as low as 60 μM almost completely inhibited specific complex formation (Fig. 3*B*). Addition of NaCl produced a gradual decrease in the binding affinity, although much higher concentrations were required (Fig. 3*C*).

Ionic Effect on H1 Binding to Mixed-Sequence Linear 146-bp DNA. We have shown that all three ions (Mg^{2+}, spermidine, and Na^{+}) can be added to levels that effectively eliminate the binding of H1 to the 4WJ DNA (Fig. 3). But what is the mechanism of this inhibition? Proteins that bind to DNA principally through ionic interactions, such as H1, can have their binding inhibited by a straightforward "salting off" effect: the protein and the salts are simply competing for binding to the phosphate backbone of the DNA, and when enough salt is present the binding of the protein is abolished. To test whether the salt concentrations that inhibit binding of H1 to 4WJ DNA are high enough to ionically displace the protein from DNA, we have studied the binding of H1 to linear 146-bp DNA derived from nucleosome core particles. DNA purified from chicken erythrocyte nucleosome core particles provided us with a convenient source of mixed-sequence DNA of a fairly well-defined length. H1 readily formed stable, soluble complexes with 146-bp DNA under many different binding conditions (G.P.S., unpublished results). The gel shown in Fig. 4 displays some typical results of the types of complexes formed between H1 and core-particle-length DNA molecules. This gel compares the pattern seen when 146-bp DNA was titrated with H1 with the patterns obtained after incubation in buffer with or without 10 mM $MgCl_2$. It is clear that although this very high level of $MgCl_2$ weakened the binding slightly, it did not eliminate it, in contrast to the situation with the 4WJ. Gels run with slightly higher H1/DNA ratios showed that, in fact, the formation of insoluble complexes of H1 with 146-bp DNA always occurred at equivalent H1 concentrations, irrespective of the presence of millimolar amounts of Mg^{2+} (data not shown). These results indicate that Mg^{2+} ions were not simply displacing H1 from DNA and that even in the presence

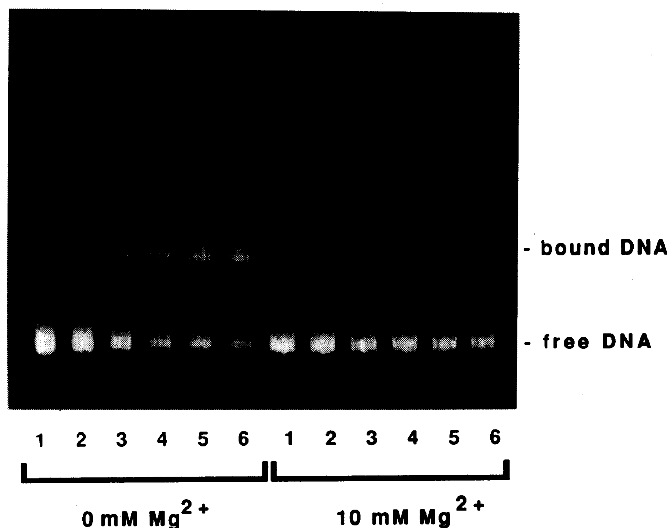


FIG. 4. Effect of $MgCl_2$ on the binding of histone H1 to linear, mixed-sequence 146-bp DNA. This gel shows a titration of 146-bp mixed-sequence DNA with histone H1; on the left no $MgCl_2$ was added, on the right 10 mM $MgCl_2$ was added to the binding reaction mixtures. Each lane contained 1 μg of total DNA. Lanes 1 contained free DNA (no protein); lanes 2–6 contained increasing amounts of H1 at increments of 0.15 μg per lane.

of 10 mM Mg^{2+} H1 can form electrophoretically stable complexes with linear DNA.

DISCUSSION

In most respects, histones H1 and H5 behave similarly in binding to 4WJ DNA. Each will form a single specific complex in the presence of a large excess of competitive linear DNA, although histone H5 binds more strongly. A major, and still unexplained, difference in behavior is found in the large difference in relative gel shifts observed for H1 and H5. This is especially surprising, since H1 and H5 are approximately the same size, and both are strongly positively charged at neutral pH. An interesting possibility, as yet untested, is that H5 compacts the 4WJ much more than does H1. That the globular domains produce nearly equivalent gel shifts suggests that the arms of the intact histones may be involved in producing this difference.

The globular domains of H1 and H5 bind to the 4WJ DNA in the presence of an excess competitor DNA with about the same affinity as does the intact protein. This implies that the structural determinants for the specific recognition of 4WJ DNA by those linker histones lie in the globular domain. In this context, it is interesting that on the basis of x-ray diffraction studies Ramakrishnan *et al.* (22) have suggested the existence of two DNA-binding sites in GH5. It seems likely that the structure-specific binding of H1 and H5 to the 4WJ involves both of these binding sites, each of which may bind to a different arm of the junction. One aspect of the behavior of GH1 and GH5 in complexing with 4WJ DNA is particularly intriguing. Whereas the intact histone shows evidence for formation of only a single complex (at least before aggregation), the globular domains exhibits multiple, stepwise binding to the junction. We suggest that each intact histone, with its N-terminal and C-terminal arms, is capable of "occupying" enough of the junction structure to allow only a single specific complex. On the other hand, the globular domains can apparently fit into two or more potential binding sites without any mutual interference.

We find that the cations Mg^{2+}, spermidine, and Na^{+} (the last at much higher concentrations) strongly inhibit H1 from binding to the 4WJ DNA. Comparable concentrations of Mg^{2+} have almost no effect on the interaction of H1 with linear 146-bp DNA. It is intriguing that very similar concentrations of these cations have also been shown to affect the conformation of 4WJ DNA in a well-characterized manner (13). There are at least two possible explanations for the effect of these cations on the binding of H1 to 4WJ DNA. H1 may recognize specifically the structure of 4WJ DNA present in the absence of cations [extended, planar as described by others (13, 23–25)] but may not be able to bind to the stacked, folded X-shaped structure that is observed in the presence of higher concentrations of these cations. Alternatively, in the absence of cations, the binding of H1 to the junction may be favored by the high phosphate charge density in the center of the junction and, thereby, allow the coaxial stacking of helical arms. In the presence of cations, this effect would be absent. We do not believe, however, that this is a simple ionic competition, for Mg^{2+} is much less effective in inhibiting the nonspecific binding of H1 to linear DNA of comparable size.

4WJ DNA may be a very useful model system for studying the interactions of proteins with internucleosomal linker DNA, specifically the DNA crossovers which are present at the entry and exit points of DNA on the nucleosome. Although this system excludes interactions of the linker DNA-binding proteins with the core histones and/or the core DNA, this may actually facilitate the analysis of other important binding determinants such as the angle of the crossover or perhaps the effects of specific sequences on the binding. Crosslinking and DNA footprinting studies may also give further insights into the interaction of the linker proteins with junction DNA.

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