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Protein D1 preferentially binds A+T-rich DNA *in vitro* and is a component of *Drosophila melanogaster* nucleosomes containing A+T-rich satellite DNA

(hybridization mapping of nucleosomal DNA/DNA binding to immobilized proteins)

LOUIS LEVINGER AND ALEXANDER VARSHAVSKY

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT **Our previous work [Levinger, L. & Varshavsky, A. (1982) *Cell* 28, 375–385] has shown that D1, a 50-kilodalton chromosomal protein of *Drosophila melanogaster*, is specifically associated with isolated nucleosomes that contain a complex A+T-rich satellite DNA with buoyant density of 1.688 g/ml. We show here that D1 is also a component of nucleosomes containing a simple-sequence, pure A+T satellite DNA, buoyant density 1.672 g/ml. Furthermore, using a modification of a protein blotting technique in which proteins are not exposed to dodecyl sulfate denaturation, we have found that D1 preferentially binds to A+T-rich double-stranded DNA *in vitro*, and it is apparently the only abundant nuclear protein in cultured *D. melanogaster* cells that possesses this property. Synthetic poly[d(A-T)]·poly[d(A-T)] and poly(dA)·poly(dT) duplexes effectively compete *in vitro* with A+T-rich *D. melanogaster* satellite DNAs for binding to D1, whereas total *Escherichia coli* DNA is an extremely poor competitor. These findings strongly suggest that D1 is a specific component of A+T-rich, tandemly repeated, heterochromatic regions, which constitute up to 15–20% of the total *D. melanogaster* genome. Possible functions of D1 protein include compaction of A+T-rich heterochromatin and participation in microtubule–centromere interactions in mitosis. In addition, D1 may prevent nonspecific binding to A+T-rich satellite DNA of other nuclear proteins that have a preference for AT-DNA, such as RNA polymerase or regulatory proteins, and may also participate in the higher-order chromatin organization *outside* tandemly repetitive regions by binding to nonrandomly positioned stretches of A+T-rich DNA.**

A nuclear protein called D1, rich in both basic and acidic amino acids, with an apparent molecular weight of about 50,000, has recently been purified from both *Drosophila melanogaster* embryos and established cell lines (1–3). Molar content of D1 in the nucleus is about 10% of the histone H1 content (1–3). A considerable proportion of D1 can be extracted from nuclei with 0.35 M NaCl, which does not remove core histones but extracts some histone H1 and most of the high mobility group (HMG) proteins (2). Apparent counterparts of D1 protein are detectable in other *Drosophila* species (2). Recent immunofluorescence analysis with anti-D1 antibodies suggested that D1 is preferentially associated with A+T-rich heterochromatic regions in *D. melanogaster* polytene chromosomes (2).

We have recently shown by two-dimensional hybridization mapping of nucleosomes that the *D. melanogaster* nucleosomes that contain a complex A+T-rich satellite DNA [1.688-g/ml buoyant density satellite with a 359-base-pair (bp) tandem repeat (4, 5)] are modified by the addition of D1 protein (3). Furthermore, core mononucleosomes that contain satellite DNA of buoyant density 1.688 g/ml (hereafter referred to as 1.688 density satellite DNA), in striking contrast to bulk chromatin, are virtually devoid of ubiquitin-H2A semihistone (uH2A), a specific covalent conjugate of histone H2A and another small protein, ubiquitin (3, 6). Selective association of D1 protein with 1.688 density satellite nucleosomes could be due to preferential D1 binding to specific nucleotide sequences within the 1.688 density satellite DNA. The 359-bp tandem repeat of the 1.688 density satellite is 69% A+T and contains several pure A+T tracts up to 14 bp in length (4, 5).

We show here that isolated nucleosomes that contain a different, simple-sequence, pure AT-satellite DNA [1.672 density DNA, with a repeating "consensus" sequence A-A-T-A-T (5, 7)] are even more tightly associated with D1 protein and that D1 displays a high preference for binding to A+T-rich double-stranded DNA *in vitro*. These latter results were obtained by a modification of a protein fractionation/blotting technique (8) that avoids exposure of proteins to strong ionic detergents and may therefore be useful for a number of other applications.

Thus D1 is an abundant, sequence-specific DNA-binding protein that binds a diverse family of tandemly repeated, heterochromatic chromosomal elements *in vivo*. Moreover, a nonrandom distribution of relatively short ($\approx 10^3$ bp) A+T-rich DNA tracts throughout the mammalian and *Drosophila* genomes (9) suggests that participation of D1 in higher-order chromatin organization may not be limited to tandemly repetitive AT-heterochromatin.

MATERIALS AND METHODS

Cell Culture and Labeling. *D. melanogaster* cells [Schneider line 2-L (10)] were maintained in spinner culture between 3 and 12×10^6 cells per ml as described (3, 10).

Chromatin Preparation and Fractionation. Nuclei were isolated by lysing cells with Nonidet P-40 and soluble chromatin was prepared by digesting the nuclei with staphylococcal nuclease as described (3, 11).

Two-Dimensional Fractionation of Nucleosomes. Nucleosomes were electrophoresed in first-dimension low ionic strength 5% polyacrylamide gels followed by second-dimension electrophoresis of nucleosomal DNA in 9% polyacrylamide/sodium dodecyl sulfate gels as described (3, 11). Second-dimension electrophoresis of nucleosomal proteins was performed in acetic acid/urea gels (12) with protamine displacement (13) as described (3, 11, 14).

Two-Dimensional Hybridization Mapping of Nucleosomes. DNA from second-dimension polyacrylamide gels was electrophoretically transferred (11) to *o*-diazophenyl thioether paper (15) after denaturation *in situ* (3, 11). The hybridization probes were the 1.688 density satellite (pDM23), containing 15 or 16

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Abbreviations: bp, base pair(s); DNP, deoxyribonucleoprotein.

copies of the 359-bp repeat cloned in pSC101 (4, 5) and the simple-sequence 1.672 density satellite (aDM672.3C, about 570 bp of tandem A-A-T-A-T repeats inserted into pBR322) (4, 7). Both DNA clones were kindly provided by D. Brutlag. These clones were constructed by A·T-tailing, resulting in the introduction of a small proportion of poly(dA)·poly(dT) between the vector and the *Drosophila* DNA insert (4, 7). Hybridization conditions were as described (3, 11).

Binding of Labeled DNA to Proteins Immobilized on Nitrocellulose. Acetic acid/urea gels (12) containing electrophoretically resolved proteins were incubated with shaking at 20°C in an excess of 10 mM KCl/10 mM magnesium acetate/0.1 mM NaEDTA/0.1 mM dithiothreitol/4 M urea/10 mM Tris·HCl (pH 7.5), with three changes of buffer at 1-hr intervals, and then blotted to nitrocellulose (BA85, Schleicher & Schuell) as described by Bowen *et al.* (8). Nitrocellulose strips were rinsed with the transfer buffer lacking urea (8) and then incubated in the same buffer (50 μl/cm^2) for 1 hr in the presence of unlabeled double-stranded *Escherichia coli* DNA (125 μg/ml) sheared by sonication to an average chain length of $\approx 10^3$ bp. This solution was then replaced for 1 hr with one containing unlabeled *E. coli* DNA (125 μg/ml) together with either 1.688 density or 1.672 density cloned double-stranded satellite DNA probe (0.1 μg/ml) labeled with ^{32}P to $\approx 1 \times 10^7$ cpm/μg by nick-translation (16) without added DNase I. Nitrocellulose strips were then rinsed with several changes of the same buffer, blotted dry, and set up for autoradiography (8, 11). In some experiments, different concentrations of unlabeled *E. coli* DNA competitor were used, and other unlabeled DNAs—such as 1.688 density satellite (pDM23), 1.672 density satellite (aDM672.3C), poly[d(A-T)·poly[d(A-T)] alternating copolymer duplex (Miles), poly(dA)·poly(dT) duplex (Collaborative Research, Waltham, MA), poly(dA), and poly(dT)—were added in various amounts as competitors.

RESULTS

Nucleosomes That Contain A+T-Rich Satellite DNAs Are Associated with D1 Protein. Fractionation of nucleosomes by gel electrophoresis coupled with the second-dimension electrophoretic analysis of their protein and DNA components permits a straightforward determination of the composition of the more abundant nucleosomal species (3, 11, 17, 18). Specifically, *D. melanogaster* nucleosomes that contain D1 protein were previously shown to migrate in a defined area of the first-dimension [deoxyribonucleoprotein (DNP)] gel relative to other nucleosomal species (ref. 3; see also brackets in Fig. 1 *C–E*). Hybridization analysis of the two-dimensional DNP → DNA pattern (Fig. 1*A*) with the 1.688 density satellite DNA probe shows a dramatic enhancement of the hybridization signal in the region of nucleosomes that contain D1 protein (Fig. 1*C*; cf. Fig. 1 *A* and *E*). It can also be seen that 1.688 density satellite core (MN1) mononucleosomes are virtually devoid of uH2A semihistone (Fig. 1*C*), as has been previously discussed in detail (3).

The 359-bp tandem repeat of the 1.688 density satellite is 69% A+T and contains pure A+T tracts up to 14 bp in length (4, 5). It was therefore of interest to compare the 1.688 density satellite-specific hybridization pattern with that for a different, simple-sequence 1.672 density satellite, which is pure AT-DNA, with a tandemly repeated consensus sequence A-A-T-A-T (5, 7). The results (Fig. 1*D*; cf. Fig. 1 *A*, *C*, and *E*) show that 1.672 density satellite mononucleosomes migrate precisely within the area of the first-dimension (DNP) pattern that corresponds to mononucleosomes that contain D1 protein (see Fig. 2*B* and ref. 3).

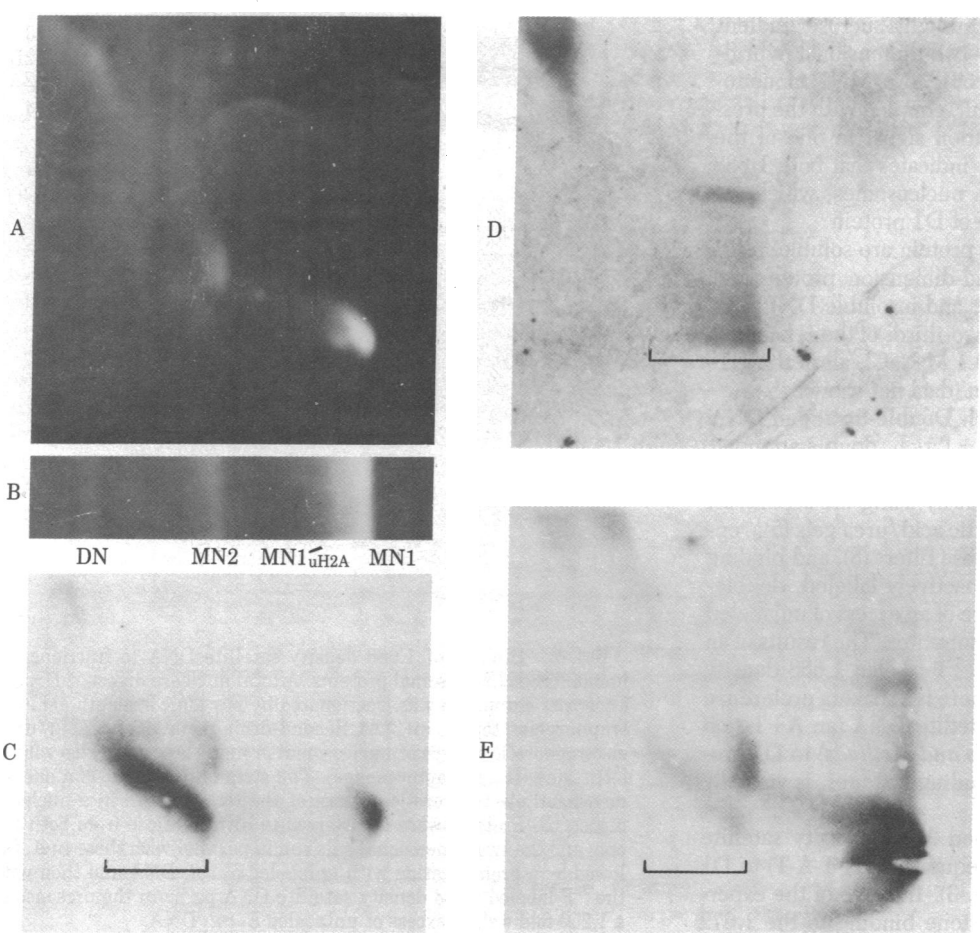

FIG. 1. Two-dimensional hybridization mapping of *D. melanogaster* nucleosomes containing A+T-rich satellite DNA. (*A*) Ethidium-stained second-dimension nucleosomal DNA pattern in a 9% polyacrylamide gel. (*B*) The corresponding first-dimension pattern of nucleosomes fractionated in a low ionic strength 5% polyacrylamide gel. (*C*) DNA from the gel in *A* was denatured by boiling *in situ*, transferred electrophoretically to *o*-diazophenyl thioether paper, and hybridized with cloned 1.688 density satellite DNA probe. (*D*) Hybridization with cloned 1.672 density satellite DNA probe. (*E*) The same *o*-diazophenyl thioether paper after removal of hybridized [^{32}P]DNA (3, 11). Brackets in *C*, *D*, and *E* indicate the first-dimension position of mononucleosomes that contain D1 protein. Nucleosome terminology: DN, dinucleosomes; MN2, mononucleosome that contains 160- to 185-bp DNA fragment, core histone octamer, and one molecule of histone H1; MN1, core mononucleosome containing 146-bp DNA fragment and core histone octamer but lacking H1, D1, and ubiquitin-H2A semihistone (uH2A); MN1$_{uH2A}$, same as MN1 but with one (or two) molecule(s) of uH2A substituting for one (or two) molecule(s) of H2A. See refs. 3, 11, and 16 for additional details.

The 1.672 density satellite-specific hybridization pattern is different in several respects from the 1.688 density satellite-specific pattern (Fig. 1*D*; cf. Fig. 1*C*), however. First, the 1.672-specific mononucleosomal DNA is of an apparently uniform size (146 bp) (Fig. 1*D*), whereas the complex 1.688 density satellite-specific DNA pattern shows both core (146-bp) and larger mononucleosomal DNA fragments (Fig. 1*C*). This difference may be due to the presence of H1 on 1.688 density satellite nucleosomes containing D1 and the absence of H1 from 1.672 density satellite nucleosomes, or to the accumulation of internal ("hidden") breaks at the core–linker junction in 1.672 density satellite nucleosomes (for a discussion of nuclease-produced internal breaks in nucleosomal DNA, see refs. 11, 17, and 19).

Second, there is a significant 1.672-specific hybridization "band" precisely above the major one (Fig. 1*D*); this area is not detected by the 1.688-specific probe (Fig. 1*C*) and contains only a trace amount of DNA in the total DNP → DNA pattern (Fig. 1*E*; cf. Fig. 1*D*). This slower-migrating 1.672-specific DNA band may arise from denaturation of some of the short (≈146-bp) fragments of the 1.672 density satellite DNA (which is pure AT-DNA) during deproteinization *in situ* before the second-dimension DNA electrophoresis (see *Materials and Methods* and refs. 11 and 17).

Third, only one of the two dinucleosomal DNA spots seen in the total DNP → DNA pattern (Fig. 1*E*) is detected by the 1.672 density satellite DNA probe, suggesting that the more rapidly migrating dinucleosomal particles are devoid of D1 protein.

Lastly, although the core mononucleosomal (MN1) DNA spot is the most prominent one in the total DNP → DNA pattern (Fig. 1 *A* and *E*), it is completely missed by the 1.672 density satellite probe (Fig. 1*D*). Removal of both D1 and H1 from mononucleosomes by centrifugation through a sucrose gradient containing 0.35 M NaCl, followed by two-dimensional hybridization analysis, does reveal 1.672-specific core (MN1) mononucleosomes (data not shown). This result, coupled with the presence of the 1.672-specific hybridization signal in one of the dinucleosomal DNA spots (Fig. 1*D*), indicates that both 1.688 and 1.672 density satellites occur in nucleosomes, which are modified, however, by the presence of D1 protein.

Mononucleosomes containing D1 protein are soluble in 0.1 M NaCl. Comparison of the second-dimension protein and DNA patterns of 0.1 M NaCl-soluble and insoluble DNP fractions shows that whereas more than two-thirds of the H1-mononucleosomes are precipitated with 0.1 M NaCl, all of the D1-mononucleosomes remain in solution (data not shown).

Preferential Binding of A+T-Rich Double-Stranded DNA by D1 Protein *in Vitro*. D1 binds A+T-rich, double-stranded DNA in the absence of nucleosomes (Fig. 2). These *in vitro* binding experiments were carried out by using the separation of proteins by electrophoresis on acetic acid/urea gels (5), replica blotting of proteins to nitrocellulose filters (8), and binding of the immobilized proteins to radioactively labeled, double-stranded DNA probes in the presence of an excess of unlabeled total *E. coli* DNA as a nonspecific competitor. The results of an experiment in which the probe was ^{32}P-labeled 1.688 density satellite DNA are shown in Fig. 2. There is a striking preference for binding of the 1.688 density satellite DNA (an A+T-rich 359-bp tandem repeat; see *Materials and Methods*) to D1 protein; binding to other proteins, including histones, is virtually undetectable.

Preferential binding of the cloned 1.672 density satellite DNA (with a repeating consensus sequence A-A-T-A-T) to D1 protein could also be observed (Fig. 3). In some of the experiments, significant core and H1 histone binding to the 1.672 density DNA probe were also seen (Fig. 3*A*, lane 1). In most cases histone binding could be suppressed by increasing the concentration of unlabeled *E. coli* DNA competitor (Fig. 3*C*). The relative intensity of D1 binding to the 1.672 density satellite DNA did not decrease significantly upon a 100-fold increase in the concentration of *E. coli* DNA competitor, from a 100-fold to a 10,000-fold weight excess over the amount of ^{32}P-labeled 1.672 density DNA probe (Fig. 3*C*).

Conditions of protein denaturation, renaturation, and DNA binding are important for detecting the preferential binding of D1 to AT-DNA: no preferential D1 binding was observed when proteins were separated on sodium dodecyl sulfate-containing gels (data not shown). The use of an apparently milder acetic acid/urea gel electrophoresis for preliminary fractionation of proteins (Figs. 2 and 3) may therefore be useful in other applications of the protein fractionation/blotting approach. Experiments shown in Figs. 2 and 3 used the binding buffer of Jack *et al.* (20). When the buffer of Bowen *et al.* (8), which lacks di-

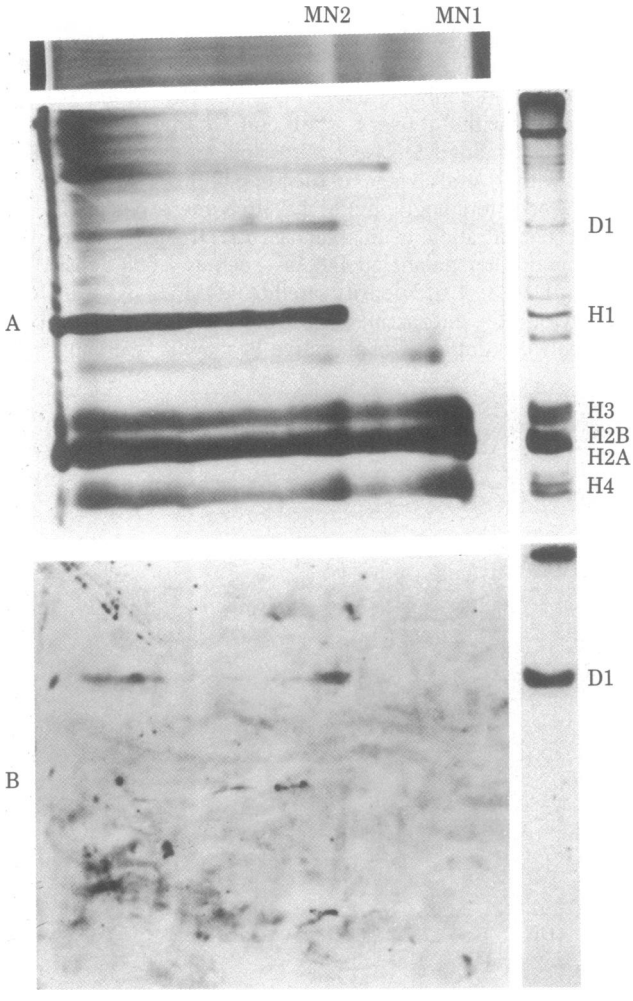

FIG. 2. Binding of 1.688 density satellite DNA to fractionated, immobilized chromosomal proteins. A total nuclease digest of *D. melanogaster* chromatin was fractionated by low ionic strength gel electrophoresis (top strip). (*A*) Second-dimension acetic acid/urea electrophoretic pattern of nucleosomal proteins labeled *in vivo* with L-[^{3}H]lysine (fluorographic image). The strip on the right is a one-dimensional electrophoretic pattern of the total protein in a nuclease digest. (*B*) Proteins were transferred to nitrocellulose from both the one- and the two-dimensional gels run in parallel with those in *A*, followed by a preincubation with unlabeled *E. coli* DNA and then with the ^{32}P-labeled 1.688 density satellite DNA probe in the presence of a 1,250-fold weight excess of unlabeled *E. coli* DNA.

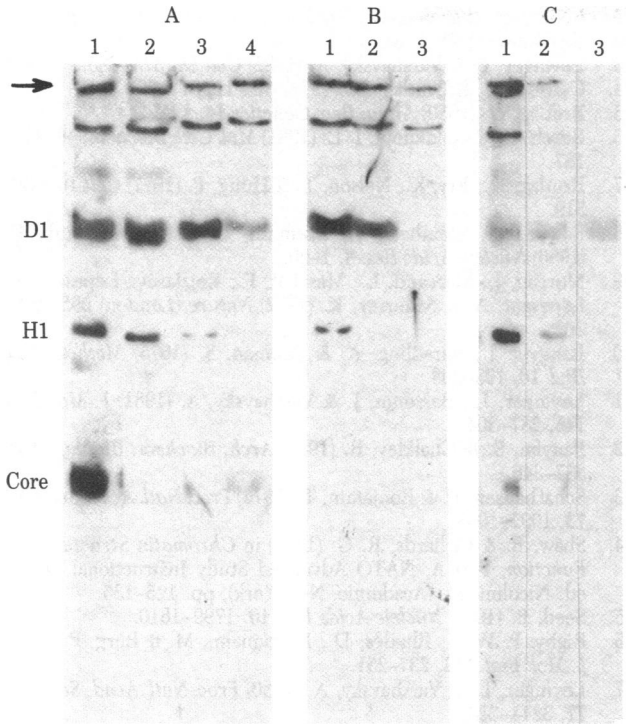

FIG. 3. Competition for binding to D1 protein between 1.672 density satellite DNA of *D. melanogaster* and other DNAs. Total chromatin proteins, separated by acetic acid/urea gel electrophoresis as in the side strips of Fig. 2, were transferred to nitrocellulose and probed with ^{32}P-labeled, nondenatured 1.672 density satellite DNA (aDM672.3C; 0.1 μg/ml) in the presence of a 1,250-fold weight excess of unlabeled *E. coli* DNA. In addition, various amounts of A+T-rich DNA competitors were added to the assay simultaneously with the [^{32}P]DNA probe. (*A*) Lanes 1–4, 1.672 density DNA competitor: unlabeled aDM672.3C plasmid was added in amounts of 0, 0.1, 1.0, and 10 μg/ml, respectively. (*B*) Lanes 1–3, poly[d(A-T)] competitor: unlabeled poly[d(A-T)]·poly[d(A-T)] was added in amounts of 0.1, 1.0, and 10.0 μg/ml, respectively. (*C*) Lanes 1–3, unlabeled *E. coli* DNA was used as a competitor in a 100-, 1,000-, and 10,000-fold weight excess over the amount of the ^{32}P-labeled 1.672 density satellite DNA probe, respectively. Positions of protein D1, core histones, and H1 are indicated on the left. An arrow indicates the electrophoretic origin.

valent cations and contains a higher concentration of monovalent ions, was used in similar experiments, no preferential binding of D1 to AT-DNA was detected (data not shown).

Unlabeled 1.688 density satellite DNA is ineffective in competition with the ^{32}P-labeled 1.672 density satellite DNA probe for binding to D1, even when present in a 100-fold weight excess over the 1.672 density satellite [^{32}P]DNA (data not shown). Unlabeled 1.672 density DNA does compete with 1.672 density [^{32}P]DNA (Fig. 3*A*). Similar results were obtained when the 1.688 density satellite DNA was used as a ^{32}P-labeled probe, except that the unlabeled 1.672 density satellite DNA competed more efficiently with the 1.688 density satellite [^{32}P]DNA than with itself (Fig. 3*A* and data not shown). Much lower relative content of pure AT-DNA stretches in the complex 1.688 density satellite DNA than in the 1.672 density DNA (7) probably explains the lower apparent affinity of 1.688 DNA for D1 protein *in vitro*.

Synthetic double-stranded poly[d(A-T)]·poly[d(A-T)] is clearly the strongest competitor (on a weight basis) for both the 1.688 and 1.672 density DNA binding to D1 protein (Fig. 3*B* and data not shown). However, after the degree of competition is adjusted for the fact that the cloned 1.672 density DNA probe (7) is only ≈10% AT-DNA, the 1.672 density satellite DNA and the synthetic poly[d(A-T)]·poly[d(A-T)] are approximately equal as competitors for binding to D1 protein *in vitro*. Poly(dA)·poly(dT) duplex competes against D1 binding by the 1.672 density satellite [^{32}P]DNA probe with an efficiency approximately equal to that of poly[d(A-T)]·poly[d(A-T)] (data not shown). Last, poly(dA) and poly(dT) separately show no detectable competition for D1 binding with the double-stranded 1.672 density satellite [^{32}P]DNA probe up to at least a 200-fold weight excess of poly(dA) or poly(dT) (data not shown), suggesting that preferential binding of A+T-rich DNA by D1 protein is confined to double-stranded AT-DNA stretches.

DISCUSSION

The two major results of this work are that D1 protein is tightly associated with most of the isolated *D. melanogaster* nucleosomes containing 1.672 and 1.688 density A+T-rich satellite DNAs and that D1 is a highly AT-DNA-specific DNA-binding protein *in vitro*; it is apparently the only abundant nuclear protein in cultured *D. melanogaster* cells that possesses this property. Cohen and his colleagues have recently shown that anti-D1 antibodies preferentially bind to A+T-rich heterochromatic regions in *D. melanogaster* polytene chromosomes (2). Our results, taken together with their findings, strongly suggest that D1 protein is specifically bound to the 1.688 and 1.672 density satellites and probably also to other A+T-rich, tandemly repetitive chromosomal regions *in vivo* as a part of their nucleosomal structure.

The DNA target recognized by D1 protein could be a stretch of A and T residues in which the specific sequence of As and Ts within the stretch has little effect on binding. This and other aspects of D1–DNA interactions could be probed *in vitro* by DNA protection experiments with the 1.688 density 359-bp complex satellite DNA sequence, using enzymatic (21) and chemical (22) methods of DNA cleavage in the presence of D1 protein.

Hsieh and Brutlag (23) have reported the presence in extracts of *D. melanogaster* embryos of an unidentified protein that preferentially binds to 1.688 density satellite DNA *in vitro*. However, the properties of their 1.688 density DNA–protein complexes [resistance to 1 M NaCl, requirement for supercoiled DNA for complex formation (23)] differ from both nucleosome- and DNA-binding properties of D1 protein observed in the present work (see *Results* and ref. 2). Thus there may be at least two different DNA-binding proteins that recognize the same satellite DNA. This possibility is strengthened by the finding of nucleotide sequence homologies between non-AT elements of cloned yeast centromeres and non-AT portions of the 359-bp repeat in the *D. melanogaster* 1.688 density satellite DNA (24). Another potential analog of D1 protein has been isolated from rat liver cells (25). This protein, BA, binds preferentially to AT-DNA *in vitro* and by immunofluorescence analysis is localized preferentially within heterochromatin (25, 26).

Because the selective removal of D1 protein from D1-containing mononucleosomes by treatment with 0.35 M NaCl results in apparently intact core (MN1) mononucleosomal particles containing satellite DNA (data not shown), it appears that D1 is present in AT-satellite nucleosomes in addition to, rather than instead of, specific core histones. Second-dimension protein analyses of D1- and H1-containing mononucleosomes (Fig. 2 and ref. 3) suggest that both H1 and D1 may be bound to the same mononucleosomal particle. Although most of the 1.672 and 1.688 density satellite nucleosomes contain D1, the stoichiometry of D1-nucleosome complexes remains unknown; a probable number is one D1 molecule per particle. It should be mentioned in this regard that even the 1.688 density satellite, which is only 69% A+T, has at least two stretches of pure AT-

DNA 7–14 bp long in each half of its 359-bp tandem repeat (4, 5). A related unanswered question is whether cores of nucleosomes containing D1 occupy sequence-specific positions within satellite DNA repeats or whether nucleosome distributions are statistical (27) in spite of the presence of D1.

Although one *a priori* plausible function for D1 would be to induce compaction of A+T-rich, tandemly repetitive chromatin, perhaps directly or by inhibiting nucleosome-ubiquitin conjugation (3), no direct evidence is available on this point.

Another potentially important function of an abundant protein with the DNA-binding properties of D1 would be to prevent A+T-rich, tandemly repetitive DNA from acting as a nonspecific "sink" for nuclear proteins such as RNA polymerases or other site-specific proteins whose DNA-binding properties are comparable to those of *lac* or λ repressor. For instance, although *lac* repressor binds to the *lac* operator about 10^6 times more tightly than to poly[d(A-T)]·poly[d(A-T)] duplex, the binding to the latter is still significant, with an association constant of 2×10^7 M^{-1} under the same solvent conditions (28). A high molar content of D1 in *D. melanogaster* chromatin (1–3) is certainly consistent with such an "AT-masking" function.

Another possibility is that relatively tight D1 binding to A+T-rich satellite DNA sequences both *in vitro* and *in vivo* masks hitherto undetected, much higher affinity DNA sites for D1 protein, analogous to the DNA-binding properties of nuclear receptors for steroid hormones (29). Because A+T-rich satellite DNAs of *D. melanogaster* are concentrated in centromeric heterochromatin (5, 30, 31), it is possible that D1 also participates in microtubule–centromere interactions in mitosis.

Last, the results of recent electron microscopic studies with partially denatured high molecular weight DNA suggest that the bulk DNA in eukaryotes contains A+T-rich stretches several hundred bp long spaced at intervals of 10–40 kbp (9). Analysis of DNA sequences in several *D. melanogaster* genes reveals that A+T-rich DNA stretches flank relatively G+C-rich DNA of the corresponding transcriptional units (9, 31, 32). These findings, taken together with our data and observation of a weak but apparently D1-specific staining of many different bands throughout polytene chromosomes by anti-D1 antibodies (2), suggest that D1 might also participate in the higher-order chromatin organization *outside* tandemly repetitive regions, through binding to nonrandomly positioned stretches of A+T-rich DNA.

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