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Structural and functional properties of linker histones and high mobility group proteins in polytene chromosomes

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ABSTRACT Variants of histone H1 and high mobility group (HMG) proteins and their genes in Dipteran insects are being studied in our laboratory and have revealed differential properties of DNA binding and intrachromosomal distribution. One of the H1 variants of *Chironomus* is found only in a minority of polytene chromosome bands and differs from the other H1 proteins of the same organism by genomic organization and by an inserted structural motif, the KAPKAP repeat, that is present also in single H1 variants of other, evolutionarily remote organisms. NH_2-terminal peptides containing the KAPKAP repeat were found *in vitro* to interact with DNA, whereas no DNA interaction was observed with the homologous peptide of another H1 variant that does not contain the inserted KAPKAP repeat. We assume that H1 variants containing the KAP motif may interact with a stretch of linker DNA and package chromatin more tightly than other H1 variants. A large series of antibodies directed against different sites in all regions of the H1 molecule is being applied in studying the sites of interaction of the H1 molecule with other molecules in interphase chromatin in terms of antibody epitope accessibility. A search for insect proteins that share properties of the mammalian HMG proteins resulted in isolation and sequencing of two different HMG1 proteins and an HMGI protein. The HMG1 proteins of the midge, *Chironomus tentans*, show a differential distribution in chromosomes. The more abundant cHMG1a protein appears uniformly distributed, whereas the less abundant cHMG1b protein could be localized only in chromosomal puffs. This strongly indicates that these highly similar proteins have different functions in chromatin. The *Chironomus* HMGI protein and the intron/exon organization of its gene were found to be very similar to human HMGI/Y proteins that are highly abundant in rapidly proliferating cells. Common properties of HMG1 and HMGI proteins include high affinity interaction with AT-rich DNA, irregular DNA structures, and the capacity to bend DNA. These properties suggest that the HMG proteins may have an architectural role in assembling different types of chromatin.

KEY WORDS: *histone H1, HMG1, HMGI, chromatin, DNA-binding*

Introduction

Influences of chromosome structure on the control of gene activity have been observed for a long time. To name an example from *Drosophila*, the position effect of "heterochromatin" onto genes in neighbouring chromosome segments, that yields patterns of two types of cell clones in which these genes are expressed or not expressed, respectively, is generally interpreted in terms of properties of chromosome structure. Recently, the isolation and cloning of genes acting as enhancers or suppressors of variegation of the position effect has revealed homologies with genes encoding nuclear proteins. The *Su(var)3-7* protein is a novel zinc finger protein possibly involved in packaging stretches of DNA (Reuter *et al.*, 1990; Reuter and Spierer, 1992). The *Su(var)3-9* protein (Tschiersch *et al.*, 1994) contains the chromo domain known from Polycomb, a protein present in many chromosome sites (Zink and Paro, 1989) that acts by repressing homeotic genes in part of the segments of the *Drosophila* embryo (Paro and Hogness, 1991), and from the heterochromatin protein, HP1 (James and Elgin, 1986). Tschiersch *et al.* (1994) have also identified in the *Su(var)3-9* protein a sequence homologous to a sequence in the *trithorax* gene (Mazo *et al.*, 1990) that encodes a putative DNA-binding protein and positively regulates homeotic genes (Lewis, 1978). Antibodies prepared against *Su(var)3-9* peptides (Steuernagel, unpublished) have recently enabled Ebensen (unpublished) in our laboratory in collaboration with Tschiersch and Reuter to show that Su(var)3-9 appears in nuclei of early stages in *Drosophila* development.

In contrast, the proteins of chromatin in a narrower sense, the histones, are usually envisaged as uniform constituents of all types of chromatin in all chromosome regions. Another quantita-

Abbreviations used in this paper: HMG, high mobility group.

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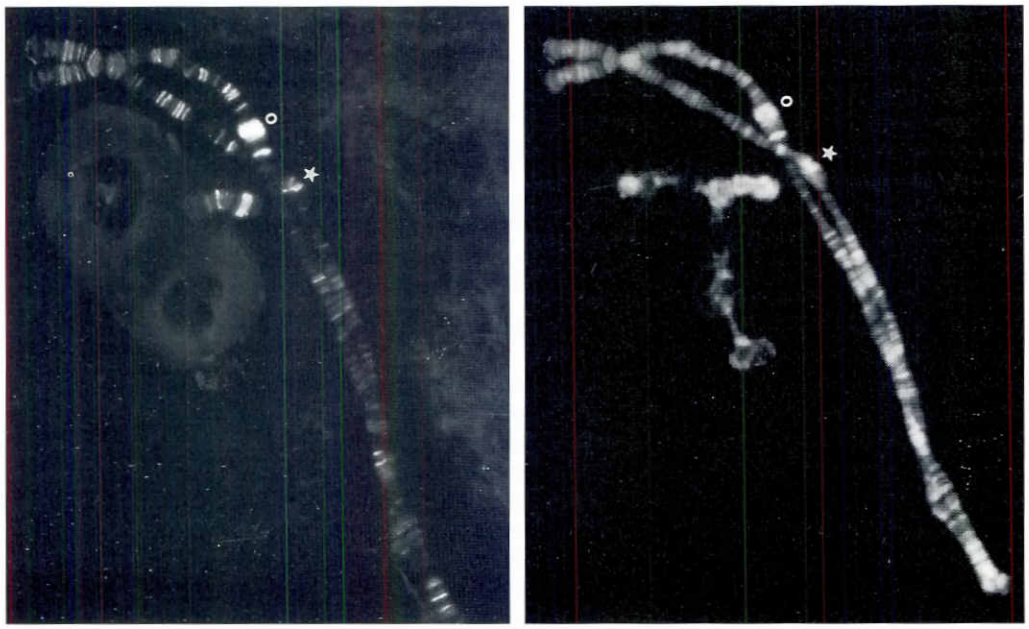

Fig. 1. Differential distribution of the histone H1 variant 1-1 in the chromosomes of a hybrid of *Chironomus thummi thummi x C. thummi piger*. *Indirect immunofluorescence with an antibody specific for H1 I-1 (left) and DNA staining by Hoechst 33342 (right) of chromosomes I and IV. The homologous chromosomes are somatically paired over part of their length; in chromosome 1, the centromere of the thummi thummi (circle) and the thummi piger (asterisks) homologs are marked. The chromosomes of the subspecies C. th. thummi contain a larger number of chromosome bands that react with the antibody. Photograph: L. Trieschmann.*

tively prominent group of chromosomal proteins, the high mobility group (HMG) proteins, contains members that are apparently distributed uniformly within chromosomes (Wiśniewski and Schulze, 1992), whereas other HMG proteins appear to be associated with transcriptionally active chromatin (Ghidelli, Thies and Wiśniewski, unpublished).

We want to understand the roles of specific chromosomal proteins in establishing the architectural environment of DNA that occurs in transcriptionally inactive, competent, and transcriptionally active chromatin. Gene regulation by epigenetic factors such as the chromatin architecture and its alterations is an important but only little understood part of development (see Davidson, 1994). In the last years, our interest has been focused on the differential intranuclear distribution of chromatin proteins, including sequence variants of histone H1 and the HMG1 proteins as well as other HMG proteins, and on the biological functions of these proteins. As model systems we are using the Dipteran insects *Chironomus* and *Drosophila* because of the cytologic and genetic advantages these organisms provide. The following report is a short review of part of our recent results on H1 sequence variants and HMG proteins and their genes.

Differential distribution of structurally divergent H1 variants in chromosomes

In the 30 nm fiber of eukaryote chromatin, the folding of the nucleosome chain is organized by the binding of histone H1 to the nucleosomes (Thoma *et al.*, 1979). Many organisms and cell types that have been analyzed carefully contain several different subtypes (sequence variants) of H1, but this heterogeneity has earlier not been considered a potential for functional diversity within the chromatin of a chromosome. *In vitro*, H1 is a repressor of transcription, and its association with nucleosomes is lost or altered during transcription (reviewed in Patient and Allan, 1989; Grunstein, 1990). However, the immunodecoration of giant chromosome puffs (Balbiani rings) *in situ* with anti-H1 antibodies and subsequent ultrastructural analysis has revealed the presence of H1 molecules in the chromatin of genes with very high transcriptional activity (Ericsson *et al.*, 1990). It should therefore be considered that certain H1 variants may repress transcription efficiently, while others may interfere less or not at all. A prerequisite for such mechanisms of epigenetic differential control of transcription to occur would, of course, be a differential spatial (or temporal) distribution of H1 variants within chromatin. Specific monoclonal antibodies have revealed that H1 variants are in fact differentially distributed in chromosomes (Mohr *et al.*, 1989). Furthermore, we find that an H1 variant restricted to or very much enriched in condensed, late replicating chromosome loci contains a novel motif that is lacking in other H1 variants and that interacts with DNA *in vitro* in a specific way (Schulze *et al.*, 1993).

The partial replacement of histone H1 by other linker histones is a conspicuous event in erythrocyte maturation (Neelin and Butler, 1961; Moss *et al.*, 1973; Tsai and Hnilica, 1975) and mammalian spermiohistogenesis (Kistler and Geroch, 1975; Shires *et al.*, 1975; Branson *et al.*, 1979). Histone H5 and histone H1t, respectively, are correlated with a higher degree of chromatin condensation and with genetic inactivity. The functionally different macronucleus and micronucleus of the ciliated protozoon, *Tetrahymena*, also contain different types of linker histones (Gorovsky *et al.*, 1974; Allis *et al.*, 1979). We have therefore asked whether different subtypes of H1 also occur in different specific structures within one nucleus. As polytene chromosomes offer opportunities to study this question, we have chosen as a model organism the larvae of several species of the Dipteran genus *Chironomus*. *Chironomus* chromosomes are the largest polytene chromosomes known and can be individually isolated because they do not stick to a chromocenter. *Chironomus* larvae are commercially available in kilogram amounts, which turned out to be important for the isolation and chemical characterization of minor H1 variants.

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C. th. p.  H1-I        SDPAPEIEAPVEAAPVASPPKGK-KEKAPKAPKAPKSPKAEK-PKSDKPKKPKVAPTHPPVSEM..
C. th. th. H1-I        SDPAPEIEAPVEAAPVASPPKGK-KE---KAPKAPKSPKAEK-PKSDKPKKPKVAPTHPPVSEM..
C. th. p.  H1-II-1     SDPAVEV-APTT--PVASPAKAK-KEK--------------K-PKTDKPKKPKAPRTHPPVSEM..
C. th. th. H1-II-1     SDPAVEV-APTT--PVASPAKAK-KEK--------------K-PKTDKVKKPKAPRTHPPVSEM..
C. th. p.  H1-II-2     SDSAVDV-APTT--PVASPAKAK-KEK--------------K-PKTDKPTKPKAPKTHPPVSEM..
C. th. p.  H1-III-1    SDPAIEV-APV---PVASPAKAK-KEK--------------K-PKSDKPKKPKAPRTHPPVSDM..
C. th. th. H1-III-1    SDPAIEV-APV---PVASPAKAK-KEK--------------K-PKSDKPKKPKAPRTHPPVSDM..

C. tent.   H1a         SDPAVEV-TPAV--PVASPAKAK-KEK--------------K-PKSDKPKKPKAPRTHLPVSDM..
C. tent.   H1b         SDPAVEV-TPAV--PVASPAKAK-KEK--------------K-PKSDKPKKPKAPRTHPPVSEM..
C. tent.   H1c         SDPAVET-APV---AVASPGKAK-KEK--------------K-PKSDKPKKPKAPRTHPPVSEM..
C. tent.   H1e         SDPAQEVEAPVEAAPVASSPKGK-KE------KAPKAPKAEK-PKSDKPKKPKAAPTHPPVSEM..

C. pall.   H1e         SDPAQEVEAPVEAAPVASSPKGK-KE------KAPKAPKAEK-PKSDKPKKPKAAPTHPPVSEM..

C. dors.   H1-I        SDPAPEVEAPVEAAPVASPPKGK-KE------KAPKAPKAEK-PKSDKPKKPKVAPTHPPVSEM..

V. cart.   H1-I        SETEAAPVVAPAAEAAPAAEAPKAKAPKAKAPKQPKAPKAPKEPKAPKEKKPKAAPTHPPYIEM..
V. cart.   H1-II                        ASDAPEVKAPKAKT----------------QKKPKTAPTHPPYIQM..

C. eleg.   H1.2        SDVT----------VAETPAV-KTPT---------KAPKAPK-SKTTTEPSSKVAAAHPPFINM..
C. eleg.   H1.1        SDSA----------VVAAAVEPKVP----------KAKAAKA-AKPTKVAKAKAPVAHPPYINT..

G. sal.    H1-I        SDSAPEIETPVEEAPKAATPAKSPAKSPGR------AKKAKK-DGSDKPKKPKAIPTHPPVSEM..
G. barb.   H1-I        SDSAPEIETPVEEAPKAASPAKSPAKSPGR------AKKAKK-DGSDKPKKPKAIPTHPPVSEM..
G. barb.   H1-II       SDPAPEV-APA--AP-VASPAK--------------AKKEKK-PKSDKPKKPKAPRTHPPVSEM..
G. barb.   H1-III      SDPAPEV-ASA--VP-VASPAK--------------AKKEKK-PKTDKPKKAKAPRTHPPVSEM..

P. angul.  H1sp              PGSP---QKRAASPRKSPRKSPKKSPRKASASPRR------KAKRARA-STHPPVLEM..
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Fig. 2. Alignment of the deduced amino acid sequences of the NH_2-terminal domains of histone H1 variants of Chironomid species, *Caenorhabditis elegans* (*C. eleg.*), the alga *Volvox carteri* (*V. cart.*), and sperm H1 from the sea urchin *Parechinus* (*P. angul.*). *The inserted KAP repeats in the structurally divergent H1 variants of Chironomus thummi piger (C. th. p.), C. thummi thummi (C. th. th.), C. tentans (C. tent.), C. pallidivittatus (C. pal.), C. dorsalis (C. dors.), and in H1.2 of Caenorhabditis elegans and H1-I of V. carteri are in red/brown colour. The SPAK repeat in Glyptotendipes (G. sal.; G. barb.) H1 and the SPKK repeats in sea urchin sperm H1 are in green/blue. The central globular domains are boxed. The residues of the central domain of H1 are shadowed in grey. The C. thummi data are taken from Schulze et al. (1993) except the sequence of C. th. th. H1-III-1 which is from Hankeln and Schmidt (1991), the C. tentans data from E. Schulze (1992), the Glyptotendipes data from Schulze et al. (1994), and the Caenorhabditis sequences from Vanfleteren et al. (1988); Vanfleteren et al. (1990); and Sanicola et al. (1990). The sequences of Volvox and the sea urchin Parechinus angulosus are taken from Lindauer et al. (1993) and Strickland et al. (1980), respectively.*

An analysis of the histone H1 complement of *Chironomus thummi* revealed six or seven electrophoretic subfractions. From the amino acid compositions and from comparative peptide analyses after chemical and enzymatic cleavage we concluded that five of these subfractions could not have arisen by proteolytic degradation of others (Hoyer-Fender and Grossbach, 1988). HPLC chromatography and sequencing of peptides later revealed that one of the five subfractions is in fact a mixture of two H1 variants, whereas the other four subfractions are individual H1 proteins encoded in different genes (E. Schulze, 1992). These proteins were regularly found in small-scale 2D-electropherograms of extracts from single larvae in inbred cultures and therefore cannot arise from allelic polymorphism (Leufgen, unpublished). A corresponding electrophoretic and chromatographic analysis yielded five larval H1 fractions in *Chironomus pallidivittatus* and *C. tentans* (Hoyer-Fender, 1985; Hoyer-Fender and Grossbach 1988; Wiśniewski, unpublished).

For an analysis of the intrachromosomal distribution of the different H1 variants, a large series of monoclonal antibodies against *C. thummi* H1 was produced and characterized by ELISA and Western blots. Most of these antibodies were found to react with all H1 subtypes and also with the H1 proteins of *C. tentans*, *C. pallidivittatus*, and other Chironomid species (Mohr, 1984). Only four of our antibodies, however, cross-react with *Drosophila* H1 and only one antibody with the H1 proteins of more distant organisms including mammals.

Three of the antibodies recognized only one H1 variant of *C. thummi*, H1 I-1, whereas another one exhibited a negative specificity by reacting with all variants except H1 I-1 (Mohr *et al.*, 1989). These antibodies made it possible to analyze the distribution of H1 I-1 versus the other H1 variants of *C. thummi* in the salivary gland chromosomes by indirect immunofluorescence. H1 I-1 was found to be located in specific chromosome bands but could not be detected in the majority of chromosome sites (Mohr *et al.*, 1989, see Fig. 1). In contrast, other H1 variant(s) were localized by the H1 I-1-negative antibody in all chromosome bands that can be identified in phase contrast or by staining for DNA.

Cytogenetic diversity within the species *C. thummi* made possible a crucial control and also provided information about the nature of the chromosome bands containing H1 I-1. Larvae of the two subspecies of *C. thummi*, *C. th. thummi* and *C. th. piger*, contain different relative amounts of H1 I-1. This difference is mirrored on the chromosomal level by the number of chromo-

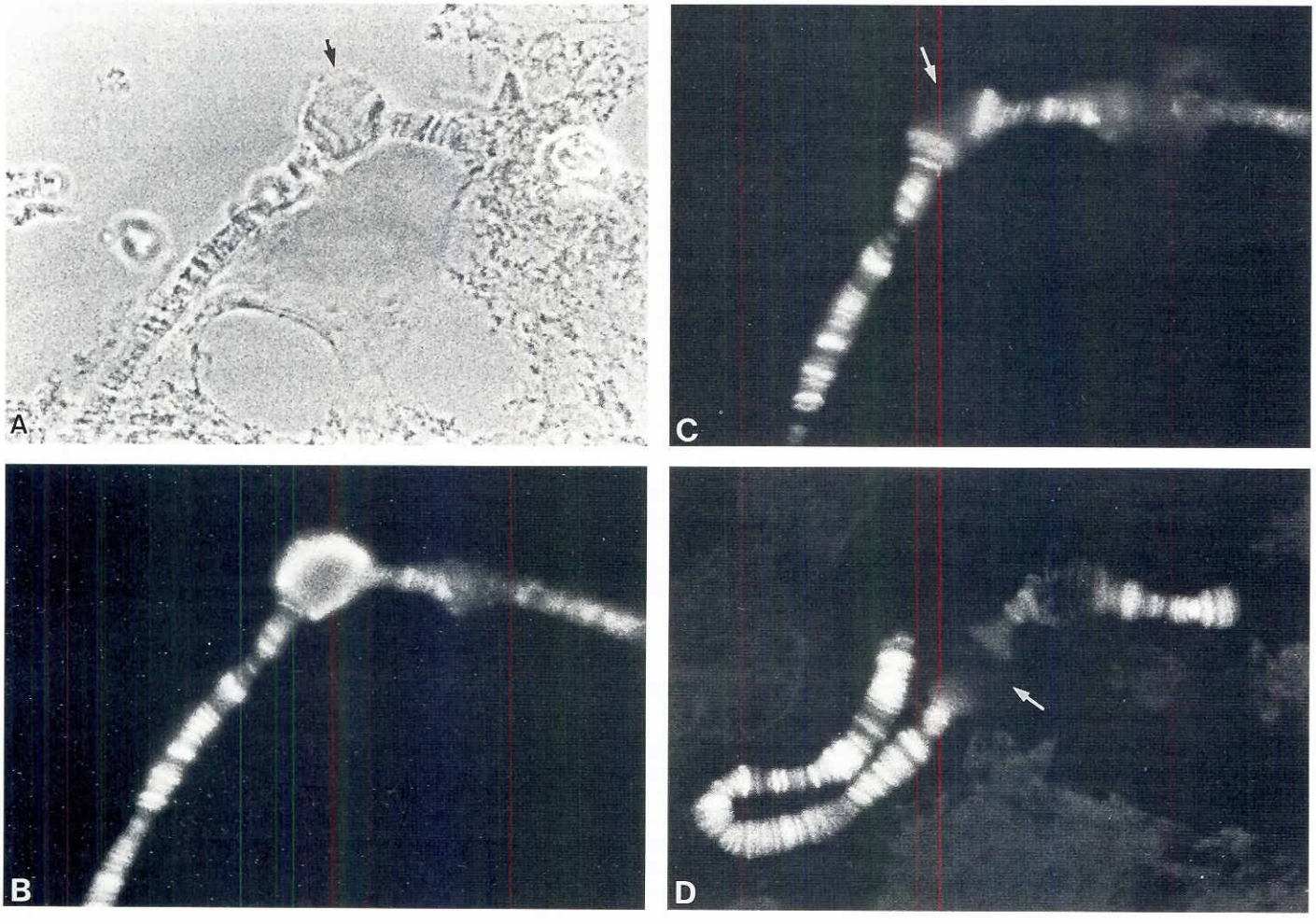

Fig. 3. Salivary gland chromosomes of *Glyptotendipes bardipes*. **(A-C)** *Part of a metacentric chromosome.* **(A)** *Phase contrast;* **(B)** *fluorescence image after staining for DNA with Hoechst 33342;* **(C)** *indirect immunofluorescence showing the distribution of a monoclonal anti-H1 antibody.* **(D)** *Immunofluorescence image of the same chromosome from another nucleus decorated with the same antibody. Note that the centromere region (arrows) is not decorated by antibody. It has, however, been shown to contain H1 by microelectrophoresis of extracts from manually isolated chromosome segments (Schulze et al., 1994).*

some bands decorated by H1 I-1-specific antibody. As is especially conspicuous in nuclei of F1-hybrids of the two subspecies (Fig. 1), bands recognized by the antibody are much rarer in the piger chromosomes than in their thummi counterparts. Lack of antibody decoration in many bands is thus not a consequence of inaccessibility of the antigen in these sites. As expected from the immunodecoration of the chromosomes of the two subspecies, extracts from F1-hybrids were found to contain intermediate amounts of H1 I-1 (Mohr *et al.*, 1989).

In the nuclei of F1-hybrids of *C. th. thummi* and *C. th. piger*, the homologues are partially somatically paired and lie in register in squash preparations (Fig. 1). Homologous chromosome bands that appear dark in *C. th. piger* but exhibit immunofluorescence with one of the antibodies specific for H1 I-1 in *C. th. thummi* are therefore easily identified. These bands show morphological differences (Keyl and Strenzke, 1956; Keyl, 1965), and most, though not all, of the bands containing H1 I-1 differ from the homologous *piger* bands by one or more of the following properties: they replicate late in S-phase and stain in a C-banding procedure (Keyl and Pelling, 1963; Hägele, 1977); they contain a higher amount of DNA and may appear more condensed (Keyl, 1965); they contain repeats of specific types of satellite DNA (Schmidt, 1984). It thus appears that the presence of H1 I-1 is characteristic of a class of chromosome bands that also have other features in common.

Two classes of H1

Cloning and sequencing of the different H1 genes of *C. th. thummi* and *C. th. piger* (B. Schulze, 1992; E. Schulze, 1992; Trieschmann, 1992) revealed the presence of two classes of H1 genes and convinced us that the first demonstration of a differential intranuclear distribution of H1 subtypes (Mohr *et al.*, 1989) is not trivial. It also led to the identification of a novel sequence motif in H1 I-1 that is evolutionarily conserved in specific H1 subtypes of organisms as distant from insects as the Nematode worm, *Caenorhabditis elegans* and the green alga, *Volvox carteri*.

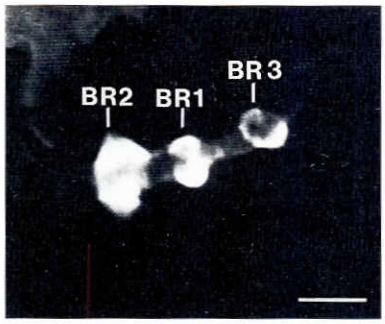

Fig. 4. Indirect immunofluorescence of chromosome IV of *Chironomus pallidivittatus* with an antibody that specifically decorates H1 in puffs. *The Balbiani rings 1, 2, and 3 (BR1, BR2, and BR3) are the most prominent puffs in this chromosome and exhibit a very high level of transcription. Photograph: E. Mohr.*

The H1 I-1 gene in both *C. th. thummi* and *C. th. piger* differs from the other H1 genes in copy number, genomic organization, and primary structure. There is one gene copy per genome in *C. th. thummi* and probably two to four copies per genome in *C. th. piger*. In contrast, the genome contains about 40 copies of the other H1 genes in *C. th. thummi* and about 50-60 copies in *C. th. piger* (Schulze *et al.*, 1993). Whereas the other H1 genes are clustered in chromosome II, the H1 I-1 genes are located in chromosome IV in both subspecies (B. Schulze, 1992; Trieschmann, 1992). Also, the sequences of the coding and 5'-flanking regions of the H1 I-1 genes differ remarkably from those of the other H1 genes (Schulze *et al.*, 1993; B. Schulze, 1992, E. Schulze, 1992; Trieschmann, 1992). We are thus dealing with two different classes of H1 genes in both subspecies of *C. thummi*. One class has only one member, the H1 I-1 gene, whereas three H1 genes, H1 II-1, H1 II-2, and H1 III-1, belong to the second class. It is important to note that two correspondingly different classes of H1 genes can also be defined in *Chironomus tentans* (E. Schulze, 1992; Nagel and Gavénis, unpublished) and the distantly related Chironomid genus, *Glyptotendipes* (Schulze *et al.*, 1994).

Most conspicuous in the histone H1 variants I-1 of *C. th. thummi* and *C. th. piger* is an inserted sequence motif that is lacking in the other H1 variants (Schulze *et al.*, 1993). It consists of a repeat of the amino acid sequence KAP, which is reiterated four times (two direct and two modified repeats) in the *thummi* and five times (three direct and two modified repeats) in the *piger* protein (Fig. 2). Another sequence element, KVA, lies 10 amino acid residues apart. In the other H1 variants, it is replaced by a single KAP. Interestingly, we have identified the motif KAPKAP-Xaa_{10}-KVA in one of the published H1 sequences of the nematode *Caenorhabditis elegans* where it lies at the same distance from a conserved sequence in the central globular domain of H1 (Fig. 2). A KAP repeat is also inserted in one of the two known H1 proteins of the green alga, *Volvox carteri* (Lindauer *et al.*, 1993) at a very similar position. The motif KAPKAPXaa_{10}-KVA is also present in one of the H1 proteins of *Chironomus tentans*, *C. pallidivittatus*, and *C. dorsalis* (Gavénis, Nagel and Schulze, unpublished; E. Schulze, 1992; see Fig. 2). Especially noteworthy is the situation in the distantly related Chironomid genus, *Glyptotendipes* (Schulze *et al.*, 1994). Three H1 genes were sequenced in *G. barbipes*, one of which was found to contain an insertion with the deduced amino sequence SPAKSPGR (Fig. 2). The same insertion is also present in an H1 protein of *G. salinus*. At their position, these insertions create the repeats SPAKSPAK-SPGR (in *G. barbipes*) and TPAKSPAKSPGR (in *G. salinus*), respectively, at a position relative to the central domain that is similar to that of the KAP motif (see Fig. 2). Both motifs are structurally related to each other and also to the SPKK repeat in the NH2-terminal domain of H1 in sea urchin sperm (Wells and McBride, 1989; Suzuki, 1989).

Nothing is known about the intranuclear distribution of these structurally divergent H1 variants, except in *C. thummi*. In *Glyptotendipes*, however, the chromosomes have large centromere regions and other sections that are very condensed and inaccessible to antibodies (Schulze *et al.*, 1994; see Fig. 3). One may speculate that this structurally divergent type of chromatin might contain the histone H1 variant with the SPAK repeat. It will also be interesting to learn whether histones in the condensed chromatin of sea urchin sperm are accessible to antibodies.

Has the presence of two classes of H1 proteins in a nucleus a functional meaning? DNA binding experiments *in vitro* have shown that the NH_2-terminal domain of *C. thummi* histone H1 I-1 competed with Hoechst 33258 for binding to DNA. This drug is known to bind to the minor groove of the DNA double-helix. In contrast, a homologous peptide from another *C. thummi* H1, that has a very similar sequence but lacks the KAPKAP insertion, did not exhibit DNA binding (Schulze *et al.*, 1993). Possibly therefore, the NH_2-terminal domain with its conspicuous insertion interacts *in vivo* with linker DNA in a specific way. It could thus e.g. contribute to establish a condensed subtype of chromatin in which transcription may be repressed.

H1 accessibility in chromatin

The higher order structure of the H1 molecule in the chromatin of interphase chromosomes and the sites of its interaction with the DNA double-helix and with other proteins are not known. As the molecular architecture of chromatin is common to all Eukaryote organisms, and in the light of accumulating evidence suggesting a major function of chromatin in gene regulation, this structure is of general interest. A novel approach to histone H1 in chromatin is the use of a large series of antibodies that are directed against different sites in all regions of the H1 molecule. These antibodies have been used as probes to check whether specific short sequences of amino acid residues in the H1 molecule, the individual antibody epitopes recognized, are accessible to antibody binding within the architecture of interphase chromatin or not. If not, this strongly suggested that the H1 molecule at this site binds to or interacts with another constituent of chromatin, or that it is hidden in an inaccessible position because such interactions occur in its neighbourhood (Steuernagel, 1994).

The analysis was performed with a series of monoclonal mouse antibodies directed against *Chironomus* H1 (Mohr, 1984; Mohr *et al.*, 1989) and a number of antibodies that are each specific for a certain epitope and that were purified from a rabbit antiserum against *Chironomus* H1 (Steuernagel, 1994). The antibodies were characterized, and their epitopes were mapped on the H1 molecule by analyzing the antibody reaction with a series of overlapping synthetic peptides that together represent the amino acid sequence of the entire H1 molecule. Each individual antibody was then tested in indirect immunofluorescence studies for its reaction with the structures of polytene chromosomes. The results (Steuemagel, 1994) with antibodies against individual epitopes showed that three specific sections within the central

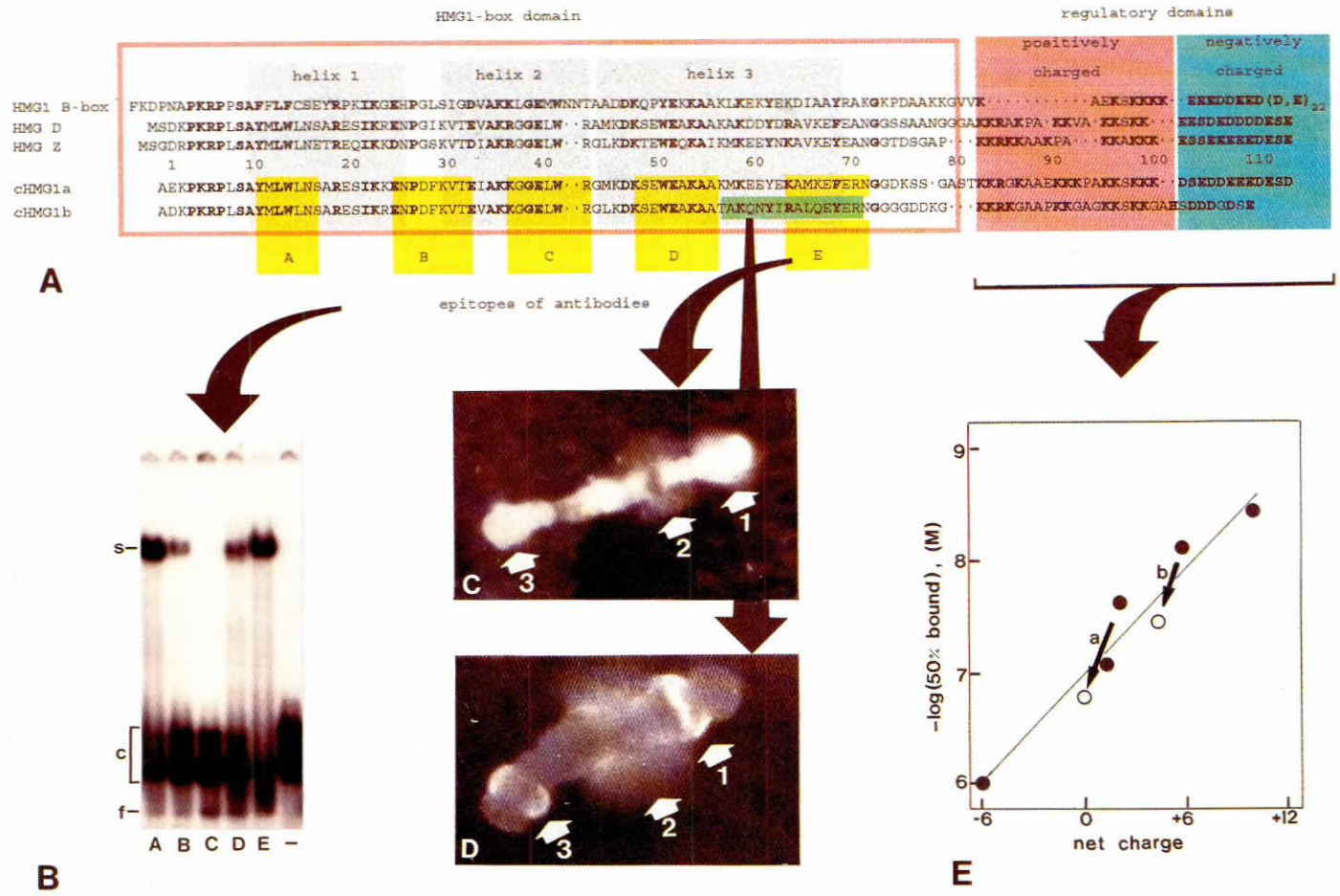

Fig. 5. Properties of insect HMG1/2 proteins. (A) *Protein sequence alignment of cHMG1a and cHMG1b from Chironomus (Wiśniewski and Schulze, 1992), HMG-D and HMG-Z from Drosophila (Wagner et al., 1992; Ner et al., 1993), and the B-domain of rat HMG1 (residues 88-215; Bianchi et al., 1989). Dots indicate gaps introduced into the sequences to maximize alignment. The numbers indicate the amino acid residues of cHMG1a. The amino acid consensus sequence is in boldface. The positions of the three helices (shadowed) of the HMG1 B-domain box structure (red frame) are from Weir et al. (1993). The positively and negatively charged COOH-terminal regulatory domains are marked by red and blue filled boxes, respectively. A peptide with a sequence corresponding to the stretch of cHMG1 b printed in green was used for production of an anti-cHMG1 b antibody. Letters A-E indicate the positions of epitopes of specific antibodies that were elicited and purified as described in Wiśniewski et al. (1994). and were used for the experiments shown in* **(B,C and D). (B)** *Antibodies directed against epitope C in helix 2 did not bind to a cHMG1a-DNA complex (for experimental details see Wiśniewski et al., 1994). In contrast antibodies recognizing epitopes A,B,D and E were able to bind to the cHMG1a-DNA complex and produced slowly migrating complexes (supershifts). This may indicate that helix 2 of cHMG1a protein is in close contact with the DNA. s, antibody-supershifted cHMG1a-DNA complexes; c, cHMG1a-DNA complexes; f, free DNA. –, lane without antibody.* **(C and D)** *Indirect immunofluorescence of squash preparations of salivary gland chromosome IV of Chironomus tentans.* **(C)** *anti-cHMG1a antibody specific for epitope E;* **(D)** *anti-cHMG1b antibody raised against the peptide marked green in* **(A).** *Arrows indicate the positions of the giant puffs (Balbiani rings 1,2 and 3).* **(E)** *The strength of interaction of the HMG1-box domain with DNA is modulated by charge of the COOH-terminal domains. The values of protein concentration at which 50% of the DNA was shifted are from Wiśniewski and Schulze, 1994; Wiśniewski et al., 1994a. Arrows show the shift in the binding strength upon phosphorylation of cHMG1a (a) and cHMG1b (b). Closed circles, unphosphorylated cHMG1b, cHMG1a, and mutants of cHMG1a; open circles, phosphorylated proteins cHMG1a and cHMG1b.*

globular domain of H1 are accessible to antibody binding. At least part of the COOH-terminal half of the molecule is also accessible. On the other hand, a series of monoclonal antibodies revealed that two short sections of the H1 molecule, the sequences DPAPE near the NH_2-terminus and SGSFK at the end of the globular domain, are only partially accessible to binding. Several of the monoclonal antibodies directed against these epitopes recognized them in chromatin whereas others did not. This suggests that these sequences in chromatin are bound to other molecules in a way that leaves part of the side chains and higher order structure accessible to antibody binding. Each series of monoclonal antibodies directed against one and the same epitope is thus a group of very subtle probes for chromatin structure at the site of the epitope (Steuemagel, 1994).

In the cell, chromatin is a dynamic structure. With antibodies as probes, it should be possible to detect alterations of this structure. This has turned out to be the case with some of our monoclonal antibodies. In all chromosomes, only a limited number of sites, which are homologous in the different nuclei, were decorated by a specific monoclonal antibody. Most conspicuous

of these are two decondensed and very actively transcribed chromosome structures, the Balbiani rings (giant puffs) 1 and 3 (Steuernagel, 1994). The sequence SGSFK near the end of the globular domain, that is recognized by the antibody, is obviously inaccessible to it in most chromosome sites, but becomes available for its binding in specific loci including the two Balbiani rings 1 and 3. This clearly indicates an alteration of chromatin structure that may, however, be rather subtle, because the epitope remains inaccessible at these chromosome loci to other antibodies. Another monoclonal antibody was found to decorate chromosome puffs including the Balbiani rings but not condensed and transcriptionally inactive chromosome regions (Mohr, 1984; see Fig. 4). Probably the epitope of this antibody is inaccessible in inactive sites of interphase chromatin but becomes accessible concomitant with decondensation and transcription. The antibody probably recognizes a conformational epitope rather than a sequence of amino acids (Steuernagel, 1994). If this is the case, the specific conformation of H1 necessary for recognition is either not present or not accessible within the architecture of inactive interphase chromatin. The antibody decoration of transcribed chromosome loci, on the other hand, would indicate a major alteration in chromatin during transcription.

The structure and DNA-binding properties of HMG1 proteins

In contrast to the histones, the evolutionary conservation of the primary structures of the HMG proteins is limited to the characteristic domains or sequence motifs. The mammalian HMG1 and HMG2 proteins have a highly conserved tripartite structure. Two folded domains A and B (the HMG-box domains) are similar to each other and are followed by a highly charged carboxy-terminal tail (Reeck *et al.*, 1979). The homologous proteins of the Dipteran insects *Chironomus* and *Drosophila*, have only one folded domain, instead of two, that is followed by a highly charged COOH terminal region of about 30 amino acid residues length (Wiśniewski and Schulze, 1992; Wagner *et al.*, 1992). The HMG-box domains of *Chironomus* cHMG1a and cHMG1b exhibit 95% and 82% sequence identity with *Drosophila* HMG1 (HMG-D), respectively (Fig. 5A). The HMG1-box domains of these proteins also exhibit a high degree of sequence identity with the HMG1-box B of mammalian HMG1 proteins (Fig. 5A).

Our binding studies, in which a series of NH_2- and COOH-terminally truncated forms of cHMG1a protein was used, revealed that the folded domains of insect HMG1 proteins are responsible for the contacts with DNA (Wiśniewski and Schulze, 1994), thus resembling the situation in mammalian HMG1 (Bianchi *et al.*, 1992). Recent NMR-spectroscopic studies have shown that the folded domain (HMG1-B box) of rat HMG1 has a L-shaped structure (Weir *et al.*, 1993). There are three helices with an angle of about 80° between the antiparallel helices 1 and 2 and helix 3. The rat HMG1-B box domain exhibits a high degree of identity with cHMG1a and cHMG1b, and it therefore it appears very probable that the *Chironomus* HMG1-box proteins have similar secondary and tertiary structures. As no structural information on HMG1-DNA complexes was available, we have identified regions of insect HMG1-boxes that are in close contact with the DNA. Using five polyclonal antibodies directed against different regions of the HMG1-box (Fig. 5B), we found that helix 2 of the HMG1-box domain of cHMG1a and cHMG1b proteins is a principal site of contacts between the protein and DNA (Wiśniewski *et al.*, 1994). Recent experiments have revealed that antibodies elicited against the highly conserved NH_2-terminal portion of the *Chironomus* HMG1-box domains, are also effective in inhibiting the cHMG1 a and cHMG1 b binding to DNA (not shown), such as was observed with antibodies reacting with helix 2. In a fluorescence competition assay with the dye Hoechst 33258 that binds to the minor groove of the DNA double helix, we observed a competitive inhibition of the drug's DNA binding upon titration with a decapeptide that resembles the NH_2-terminal portion of cHMG1a (Wiśniewski, unpublished). Both observations suggest that the NH_2 terminal fragment of the domain makes contacts within the minor groove of B-DNA. However, this interaction is not crucial for the interaction of cHMG1a with either linear or cruciform DNA in general, because a the cHMG1 a mutant lacking the NH_2-terminal sequence exhibited an only 5 times lower binding strength as compared to the entire cHMG1a protein (Wiśniewski and Schulze, 1994).

A negative effect of the COOH-terminal part of HMG1 on the strength of DNA-binding has frequently been observed in vertebrate HMG1 and HMG2 proteins (Carballo *et al.*, 1983; Sheflin *et al.*, 1993). To analyze it in more detail, we have produced several protein mutants and peptides of cHMG1a and studied their DNA-binding behavior. Our data revealed that sequences flanking the folded HMG1-box domain are essential for its interaction with DNA (Wiśniewski and Schulze, 1994). In particular, changes in the numbers of positive and negative charges, respectively, within the COOH-terminal basic and acidic domains (Fig. 5A) modulated the DNA binding affinity of HMG1 protein (Fig. 5E). The alteration of fluorescence of the tryptophan residues of the HMG1-box domain suggest that this modulation is due to interaction of the acidic domain with the positively charged HMG1-box domain (Wiśniewski and Schulze, 1994).

As posttranslational modifications such as phosphorylation would provide a sensitive mechanism for regulating the binding of HMG1 proteins to DNA, we have analyzed the phosphorylation of cHMG1a and cHMG1b in an embryonic *Chironomus* cell line *in vitro*. We have found that *Chironomus* HMG1 proteins are efficiently phosphorylated by protein kinase C or another related enzyme. The phosphorylation sites were found to be located exclusively within the positively charged regulatory domain of the HMG1 proteins (Wiśniewski *et al.*, 19994a). This particular phosphorylation pattern could be achieved *in vitro* using rat protein kinase C. The modification resulted in a 5- to 10-fold reduction of the DNA-binding affinity of the HMG1 proteins (Fig. 5E; Wiśniewski *et al.*, 1994a).

Subcellular distribution of *Chironomus* HMG1 proteins

A specific feature of the HMG1/2 proteins is their variable distribution between cytoplasm and nucleus (Einck and Bustin, 1985) that depends on the cell type and the physiological state of the cell. Neither the mechanism nor the biological meaning of this process are understood. Mosevitzky *et al.* (1989) suggested that a posttranslational modification of the HMG1 and HMG2 proteins may influence their distribution between cytoplasm and

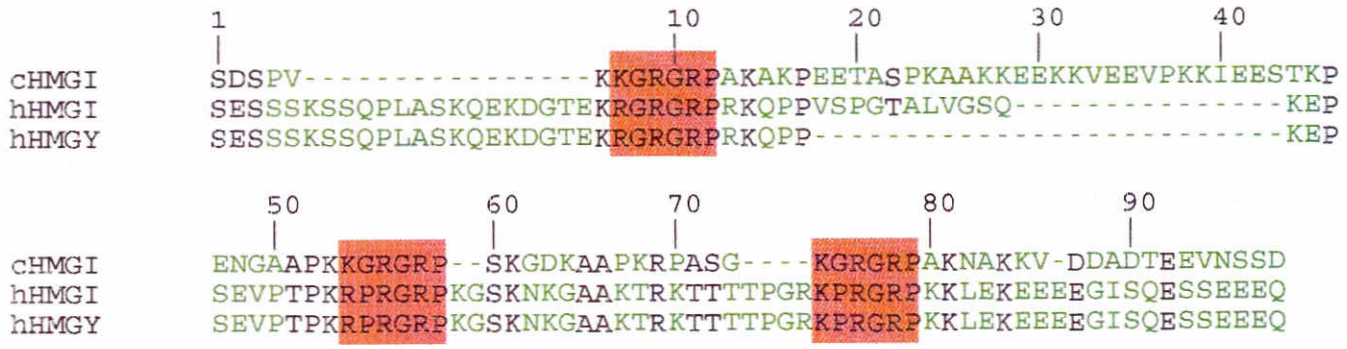

Fig. 6. Alignment of the primary structures of *Chironomus* cHMGI, human HMGI and human HMGY. *The amino acid residues which are identical or well conserved between Chironomus HMGI (cHMGI) and human HMGI/Y (hHMGI and hHMGY); Lund et al., 1987; Eckner and Birnstiel, 1989) are in black. The non-conserved residues are in green. Gaps were introduced to maximize alignment. The numbers refer to the positions of the cHMGI protein. the K/RXRGRP motifs are boxed in red.*

the nucleus. To follow this suggestion, and with respect to the observed phosphorylation of the *Chironomus* cHMG1a and cHMG1b proteins *in vivo*, we have analyzed the translocation of the HMG1 proteins from cytoplasm to nucleus in the salivary gland cells of *Chironomus* by a microinjection technique. The translocation of unphosphorylated protein was a rapid process, whereas the nuclear translocation of phosphorylated cHMG1a was inhibited (Wiśniewski *et al.*, 1994a). Our results suggest that phosphorylation/dephosphorylation may regulate the distribution of HMG1 proteins between cytoplasm and nuclei. As this distribution has been shown to vary in a specific way between different cell types and between neonatal and terminally differentiated liver cells (Mosevitsky *et al.*, 1989), phosphorylation of HMG1/2 proteins could be involved in a mechanism for establishing a type of chromatin that contain relatively little HMG1/2 and that is more frequent in terminally differentiated than in embryonic cells.

The functional significance of the heterogeneity of HMG1/2 proteins

Similar to mammalian species, where two abundant HMG1-box proteins, HMG1 and HMG2, occur two highly similar and abundant HMG1-box proteins have also been detected in a number of other organisms including insects, protozoans, and yeast. DNA-binding properties (Schröter and Bode, 1982; Wiśniewski and Schulze, 1994), cellular distribution (Seyedin and Kistler, 1979; Kuehl *et al.*, 1984; Mosevitzky *et al.*, 1989; Wang and Allis, 1992), and the comparison of expression during development (Ner *et al.*, 1993), of the two HMG1 box proteins within the same organism suggested functional difference(s).

In *Chironomus* the cHMG1a and cHMG1b proteins differ only slightly in their primary structure (81% sequence identity in the HMG-box domain, Wiśniewski and Schulze, 1992). More pronounced, however, are differences in their spectroscopic properties (Wiśniewski and Schulze, 1994) and in interaction with some antibodies (Wiśniewski *et al.*, 1994). This suggests that the proteins have subtly diverse tertiary structures, which might result in their functional diversity. Using site-specific antibodies directed against cHMG1a and cHMG1b we found that these proteins are differentially distributed in polytene chromosomes of salivary gland cells. The antibodies against the abundant cHMG1a decorated the entire chromosomes, whereas the less abundant cHMG1 b could be detected only in the decondensed chromatin of puffs (Fig. 5C and D). It thus appears that cHMG1b and cHMG1a participate in assembling different types of chromatin, respectively. Our recent studies indicate that the genes of cHMG1a and cHMG1b are differentially regulated. In *Chironomus* cells in culture that differentiate upon addition of 20-hydroxy ecdysone the concentration of cHMG1a-mRNA remained unchanged in comparison with cells not treated with the hormone whereas the number of cHMG1b transcripts was reduced by 30-40% (Claus and Wiśniewski, unpublished). Interestingly, in an evolutionary distant organism, the ciliated protozoan *Tetrahymena,* the abundant HMG1-box protein HMGB was found in both transcriptionally active macro- and inactive micronuclei, whereas the less frequent HMGC protein could be isolated only from the transcriptionally active macro nuclei (Wang and Allis, 1992). Taking all these data together, it appears plausible to assume that the two abundant HMG1-box proteins present in eucaryotic organisms play different roles in assembling particular chromatin forms.

The HMGI protein of *Chironomus*

A search for insect proteins with physico-chemical properties characteristic for the vertebrate proteins of the HMG14/17 and HMGI/Y families resulted in the isolation and sequencing of a protein exhibiting a high similarity to HMG protein I of mammals (Fig. 6). It has a deduced molecular mass of 10,371 kDa and appears to be a product of a single gene copy. Similar to mammalian HMGI/Y proteins, the insect cHMGI protein has three putative DNA-binding motifs with the sequence K/RXRGRP that are each encoded by one exon of the gene. This supports the idea of exon shuffling by insertion of exon sequences to other genes in the course of evolution (Friedmann *et al.*, 1993). The DNA binding consensus sequence K/RXRGRP is found in a number of non-HMG proteins including, for example, the methylated DNA binding protein MeCP2 of rat (Lewis *et al.*, 1992), the human HRX (Tkachuk *et al.*, 1992), and *Drosophila* D1 protein (Ashley *et al.*, 1989).

Protein cHMGI binds to AT-rich DNA with an half-saturation value of 1.1 nM. Using synthetic peptides we demonstrated that two of these motifs are necessary for high-affinity binding suggesting that intramolecular cooperativity of two (or more) K/RXR-GRP binding motifs is responsible for high-affinity binding of HMGI proteins to DNA (Claus *et al.*, 1994).

Possible functions and interaction with linker histone H1

Up to now our studies on HMG proteins in Dipteran insects, have resulted in isolation and characterization of two proteins that are highly similar to vertebrate HMG1/2 and one protein with homology to mammalian HMGI. They are highly abundant chromosomal proteins exhibiting high DNA-binding affinity to linear AT-rich DNA and to cruciform DNA (Claus *et al.*, 1994; Wiśniewski and Schulze, 1994). These properties are consistent with a structural role of these proteins. High affinity binding to cruciform DNA is also a property of the linker histones H1 and H5 (Varga-Weisz *et al.*, 1994). It is conceivable that HMGI/Y and HMG1/2 are able to compete with linker histones for their position on the nucleosomes. This could lead to the formation of different subtypes of chromatin in replicating, transcriptionally active, transcription competent, and transcriptionally inactive sections of chromosome. Our data suggest that the protein cHMG1b could be involved in the assembling of active chromatin whereas the cHMG1a plays an other unknown role. It is homologous to *Drosophila* protein HMG-D, a component of early embryonic chromatin in which histone H1 is lacking (Ner and Travers, 1994). At this developmental stage, the DNA undergoes rapid replication, and cHMG1a or HMG-D could be involved in establishing a type of *replicating* chromatin. The HMGI/Y proteins, on the other hand, are abundant only in undifferentiated and rapidly proliferating cells and might be involved in assembling another specific subtype of chromatin.

In the last five years a number of proteins have been discovered that exhibit partial primary structure similarities to the HMG1-box domain proteins and the K/RXRGRP motif of HMGI/Y proteins. Most of them function as transcriptional regulators that bind specifically to particular regulatory elements. The sequence specificity of these transcriptional regulators is limited, and binding strength for specific DNA interaction has in some cases been reported to be only 20-40 fold higher than that in nonspecific interaction (Giese *et al.*, 1991). Therefore the exclusive binding of such regulatory proteins to their target sequences would require saturation of a unrelated DNA sequences with other DNA-binding molecules. The HMG1/2 and HMGI/Y proteins are good candidates for such a role. This may also be relevant in context of saturation of distorted, damaged, or bent DNA regions conformations that are preferential sites of binding of a number of transcriptional regulators containing HMG1 box domains.

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