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Multiple phases of nucleosomes in the hsp 70 genes of Drosophila melanogaster

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ABSTRACT

The arrangement of the nucleosomes with respect to the DNA sequence has been examined in the genes coding for the major heat shock protein (hsp 70) in Drosophila. In the repressed state of the genes, the nucleosomes are precisely phased in at least three frames.

INTRODUCTION

The association of DNA with histones in chromatin to form nucleosomes has been recognized since its discovery to be a means of organizing the DNA of eukaryotes in a regular manner (1-3). The binding of the DNA to the outside of a histone octamer in nucleosomes introduces changes in the topology of the DNA double helix that may have important implications for the recognition of DNA signals. Thus, a signal may not simply be represented by a sequence of contiguous base pairs but could consist of a set of sequences widely separated on the linear DNA yet closely apposed, for example, on adjacent turns of the supercoiled DNA in the nucleosome (4). If the nucleosomes have to expose (or cover) regulatory signals, one would expect that in those regions comprising the signals the histones do not interact randomly with the DNA and may even exhibit a precise phase relation with respect to the nucleotide sequence. In this latter case, nucleosomes are said to be phased (5).

The observations that the average spacing of nucleosomes changes during replication (6) and development (7-10) and that the nucleosomal repeat disappears during transcription (11; Levy and Noll, submitted) imply that the nucleosomes cannot occupy a unique phase all the time. Early studies demonstrated that there is no single phase relation between nucleosomes and the bulk of the unique DNA sequences (12,13). While a unique frame of nucleosomes in the α-satellite DNA of African green monkey cells (14) is clearly ruled out by more recent experiments (15), studies with the SV40 and polyoma minichro-

mosome suggest that nucleosomes are not arranged in a single phase (16,17) but rather occupy a few defined frames (18). Recently, it has been shown that the nucleosomes in tRNA genes of chicken are phased uniquely (19) whereas in 5S genes more than one phase seems to exist (Louis et al., submitted; J. Gottesfeld, personal communication).

In a previous communication we have examined the structure of the genes coding for the major heat shock protein in Drosophila (hsp 70) both in the repressed and transcribed state (Levy and Noll, submitted). The nucleosomes of the repressed gene were found to be part of a protected domain comprising 2.5 kb which is less susceptible to cleavage by micrococcal nuclease than adjacent regions. Because the limits of the protected domain are defined rather accurately and the relative spacing of the nucleosomes within the domain is fairly precise (180 ± 4 base pairs with a possible variation of less than 10 base pairs; Levy and Noll, submitted), the nucleosomes might not be arranged randomly with respect to the DNA sequence of these genes. In this report we demonstrate that the nucleosomes are arranged in a small number of precise frames with respect to the DNA sequence of the hsp 70 genes in Drosophila Kc cells.

MATERIALS AND METHODS

Preparation of nuclei and digestion with micrococcal nuclease. Drosophila Kc cells (3 l) grown at 25°C to a density of 9.5×10^6 cells per ml were harvested, and nuclei were prepared and suspended in 10 ml of Mg^{2+}-buffer, 0.5 mM PMSF, 1 mM $CaCl_2$ as described (Levy and Noll, submitted). The nuclei were digested with micrococcal nuclease (1050 units/ml) at 25°C for 1 h. The DNA was extracted and treated with a mixture of RNase A and T1 RNase.

Preparation of core particle DNA. The DNA fragments of the micrococcal nuclease digest were separated in a 4% polyacrylamide gel in TBE-buffer (20). The DNA band of 146 base pairs was cut out, and the DNA was recovered by electroelution in a dialysis bag in 0.1xTBE-buffer at 4°C overnight. The DNA was extracted with phenol, precipitated with ethanol, dissolved in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and passed over a Sephadex G-100 column prepared in a pasteur pipette and equilibrated in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. The eluted DNA fractions were pooled and made 2 mM in $MgCl_2$.

Digestion of core particle DNA with restriction enzymes. The purified core particle DNA was digested with various restriction endonucleases (10 µg

of DNA per enzyme) in the buffers recommended by BioLabs, precipitated with ethanol, and dissolved in 0.25xTBE-buffer, 3% Ficoll, bromophenolblue. The restricted core particle DNA was loaded on a 4% nondenaturing polyacrylamide gel in TBE-buffer (20). After electrophoresis, the DNA was transferred to DBM-paper (21) and hybridized with 0.17 µg of the nick-translated (22) Sal-Sal fragment ($1.7x10^7$ cpm) isolated from its subclone in pBR322. The subclone was derived from the genomic clone 56H8 of an hsp 70 gene (23) and a gift of M. Goldschmidt-Clermont. Hybridization was carried out at 55°C as described (21).

RESULTS

To test whether nucleosomes are phased in repressed hsp 70 genes, we used an experimental approach based on a similar idea employed in previous schemes by other groups (12-14, 18). Briefly, nucleosome core particles containing a defined DNA size of 146 base pairs were generated by extensive digestion of nuclei with micrococcal nuclease (24-26). The DNA of the core particles was purified in a polyacrylamide gel and digested with various restriction enzymes known to cleave the hsp 70 gene. The restricted DNA was separated on a 4% polyacrylamide gel, transferred to DBM-paper with high efficiency (21), and hybridized to a radioactively labeled probe consisting of the entire transcribed portion of the hsp 70 gene. If the nucleosomes are phased and the restriction site is within a core particle, the two ends of the 146 base pairs of the core particle are at defined distances from the restriction site, and hence two DNA fragments are produced that complement to 146 base pairs. If the nucleosomes are arranged in multiple phases, multiple pairs of fragments are expected whereas no discrete bands smaller than 146 base pairs will be observed in case of a random distribution of the nucleosomes with respect to the DNA sequence.

Because the experiment does not discriminate between a random distribution of the nucleosomes along the DNA and the location of the restriction site in the linker region of phased core particles (in both instances no bands below 146 base pairs are visible), the core particle DNA was digested with several different restriction enzymes that cleave within the transcribed region of the hsp 70 genes. This region which was used as probe for hybridization comprises 2.2 kb or 12 to 13 nucleosomes (Levy and Noll, submitted). Since the DNA of only one of the 13 core particles that hybridize to the radioactive probe will be cleaved into a pair of fragments if there is a single

phase, merely 7% of the hybridizing radioactivity is expected in each restricted DNA fragment. The restricted DNA fragments will hybridize to an even lower percentage of the radioactive probe if the nucleosomes are arranged in several phases or if the restriction site is not present in all six hsp 70 genes. Thus, for example, after restriction with Bgl I which cuts in two of the six hsp 70 genes (27), the DNA bands below 146 base pairs will contain less than 2.4% of the radioactivity.

The results of such an experiment are shown in Figure 1. In the left panel, a small number of weak bands of discrete sizes are visible below the overexposed main band of 146 base pairs after digestion with the restriction enzymes Bam, Bgl I, or Sal. No such bands are present in the control (c), in which the DNA has not been restricted, or after digestion with Xba. The main band of 146 base pairs appears to be contaminated with a small amount of larger DNA fragments trailing behind (control in left panel of Fig. 1). The

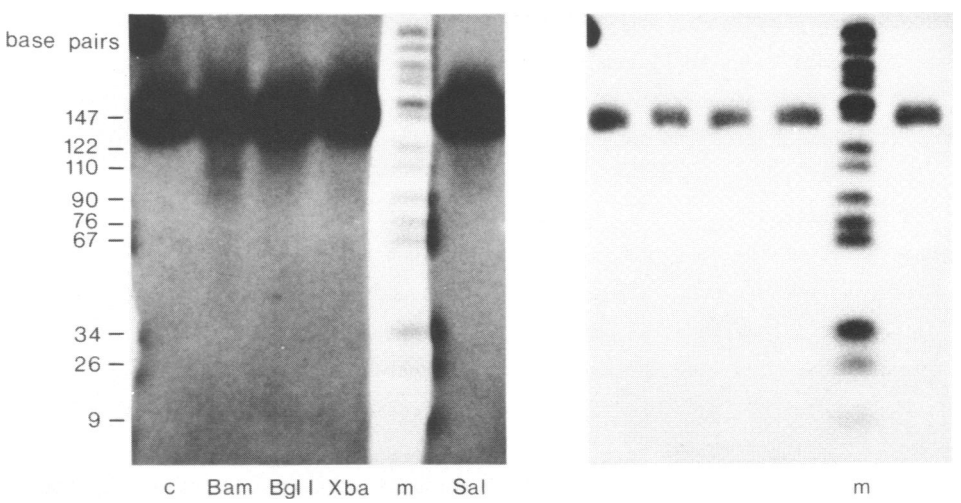

Figure 1: Analysis of restricted core particle DNA of the hsp 70 genes. Core particle DNA before (c) and after digestion with the restriction endonuclease Bam HI (Bam), Bgl I, Xba I (Xba), or Sal I (Sal) was separated by polyacrylamide gel electrophoresis and transferred to DBM-paper. For calibration, Hpa II fragments of pBR322, labeled at their 3'-ends with ^{32}P-dCTP by "Klenow" DNA polymerase I, served as markers (m). The DNA bound to the paper was hybridized to the nick-translated Sal-Sal fragment consisting of the transcribed portion of the hsp 70 genes (23). The paper was autoradiographed for a short time (right panel) and for a time corresponding to 25 times more desintegrations (left panel). To avoid overexposure, the marker lane in the left panel was covered with a lead strip during autoradiography.

possibility that the restricted DNA bands below 146 base pairs are derived from these DNA fragments was ruled out by scanning the autoradiogram with a Joyce-Loebl densitometer. The scans demonstrate that no reduction compared to the control of the DNA trailing the main band of 146 base pairs is observed after restriction (not shown). A much shorter exposure shown in the right panel proves that the core particle DNA is in a sharp band of 146 base pairs. Hence, the weak bands observed are derived from a single DNA fragment length the ends of which are defined solely by the structure of the nucleosome core particle. It follows that the nucleosomes are arranged in a few precise frames with respect to the DNA sequence of the hsp 70 genes.

The sizes of the DNA bands obtained after cleavage with Bam, Bgl I, or Sal have been calibrated with marker DNA (labeled "m" in Fig. 1), a Hpa II digest of the plasmid pBR322 (Fig. 2). Three Bam fragments (119, 106, 94 base pairs), two Sal fragments (114, 98 base pairs), and one Bgl I fragment (111 base pairs) are observed. The smallest visible fragment has a size of 94

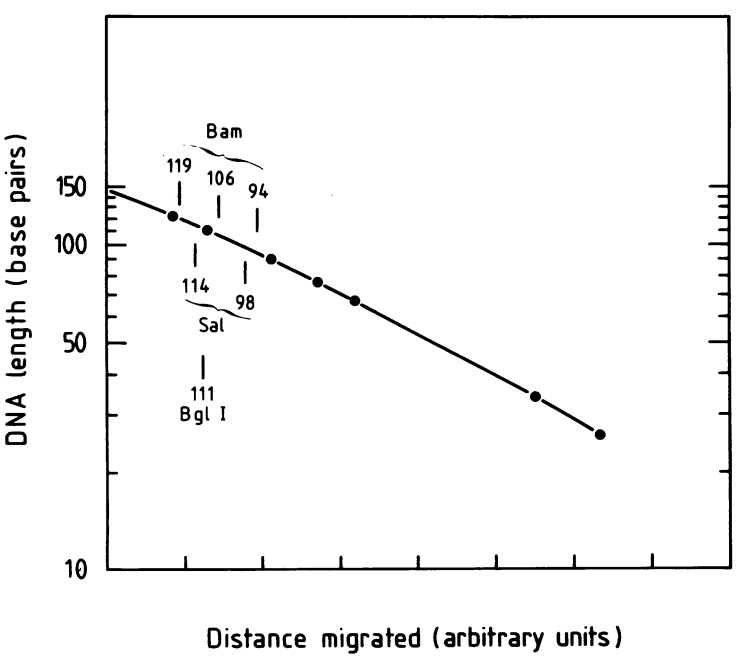

Figure 2: Size calibration of restricted core particle DNA of hsp 70 genes. The lengths of the restricted DNA fragments shown in Figure 1 have been calibrated with a Hpa II digest of pBR322 (28).

base pairs and thus no pairs of fragments complementing to 146 base pairs are apparent. The reason why the complementary fragments of 52 base pairs or less are not detected is the drastic reduction of the hybridization efficiency with decreasing DNA size below 80 base pairs (21).

Because the signal of the restricted DNA is only a few percent of that of the intact core particle DNA, the latter was overexposed, resulting in a broad band (left panel of Fig. 1). Hence, restricted fragments longer than 120 base pairs would not be resolved from the main band. Therefore, we cannot exclude additional phases of the nucleosomes that would generate restricted DNA sizes between 120 and 146 base pairs. Obviously, phases that place the restriction site into the linker region are not detected either. In fact, the absence of bands after cleavage with Xba (Fig. 1) locates this restriction site within or at a distance of less than 25 base pairs from a linker region. The observation of only one band after Bgl I digestion raises the possibility that the nucleosomes are perfectly phased in the two copies of the hsp 70 gene containing this restriction site (27).

The restriction map of the hsp 70 genes (23) shown in Figure 3a is known precisely from the DNA sequence (29, 30). The Sal site at -12 base pairs from the putative start of the transcription (29) and the Bgl I site are present only in two copies of the six hsp 70 genes (23, 27). These two genes are located at 87A7 in wild type flies whereas the remaining four copies lacking the Bgl I and the left-hand Sal site are present at 87C1 (31, 32). The arrangements illustrated in Figure 3b of the nucleosomes with respect to the DNA sequence of the hsp 70 genes are consistent with the restriction map, the average nucleosomal repeat of 180 $\pm$ 4 base pairs in hsp 70 genes (Levy and Noll, submitted), and the lengths of the restricted core particle DNA (Fig. 2). The three Bam fragments of 94, 106, and 119 base pairs indicate that there are at least three phases. At present, we cannot unequivocally assigne the Sal and Bam fragments and the Bgl I fragment to one phase for several reasons: (i) the average nucleosomal repeat of 180 base pairs may be off by 4 base pairs (Levy and Noll, submitted), (ii) the lengths of the linkers may not be constant in the gene, (iii) additional fragments larger than 120 base pairs which would be obscured by the overexposed main band DNA could exist, and (iv) distances between restriction sites might vary slightly in different gene copies (e.g. a copy at 87A7 exhibits an Xba-Sal distance of 315 base pairs (29) whereas in another copy at 87C1 the Xba-Sal distance is

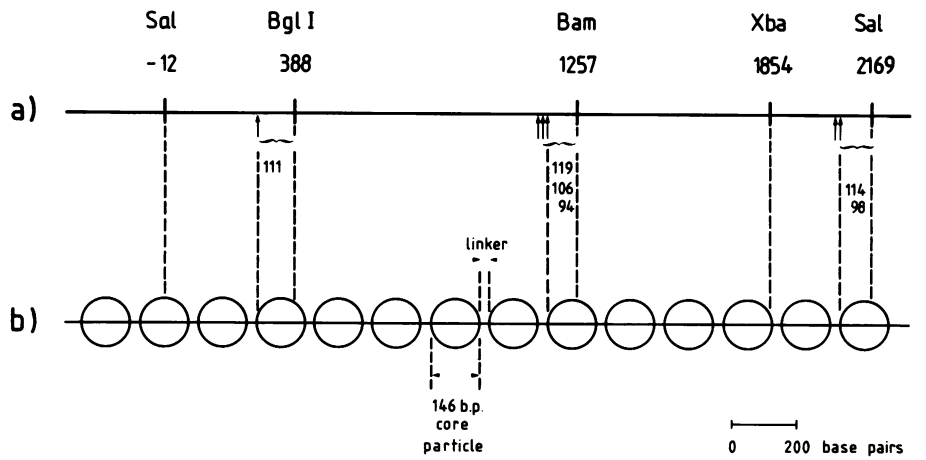

Figure 3: Phasing of nucleosomes in hsp 70 genes. (a) Restriction map of hsp 70 genes (23). The large numbers represent distances from the putative cap site in base pairs and are known from the DNA sequence of the gene (29, 30). The four gene copies at 87C1 lack the Bgl I and the left Sal site (31). Arrows and small numbers indicate the boundaries of the nucleosome core particles and their distances (in base pairs) from the restriction sites. (b) Phase relation of nucleosome core particles with respect to DNA sequence of hsp 70 genes. The positions of the nucleosome core particles in relation to the restriction map are illustrated schematically. Only one arrangement of the three phases compatible with the lengths of the restricted core particle DNA is shown.

only 312 base pairs (30; F. Karch, personal communication)). Because of these uncertainties it is possible that in some phases the orientation of the nucleosomes at the Bgl I and Bam site is opposite to the one shown in Figure 3b. In addition, the observed Sal fragments cannot be assigned unambiguously only to the right Sal site.

The results show, however, that the nucleosomes are precisely phased in at least one phase in the hsp 70 genes of one locus and in at least two phases in the hsp 70 genes at the other locus.

DISCUSSION

The approach we have chosen to test whether nucleosomes are phased in hsp 70 genes has two important advantages. First, all DNA fragments before the digestion with restriction enzymes are of the same size which is determined solely by the structure of the nucleosome core particle. This avoids

ambiguities of interpretation that arise either when the fragments used are of heterogeneous size or when they are of a unique length that is not determined only by the nucleosome structure. Such fragments may arise after mild digestions due to a preference of micrococcal nuclease to cleave after A-T base pairs (33) or at specific sites exposed by nonnucleosomal factors. Second, our approach provides an analysis at high resolution and thus permits a precise determination of the phases. In the hsp 83 gene, for example, what at a lower resolution may appear to be a single phase (34) might well turn out to consist of multiple phases if examined with the precision employed here for the analysis of the hsp 70 genes.

We do not know whether there is only one phase in each copy of the six hsp 70 genes in the genome of Kc cells (27) or whether several phases exist in some or all of the hsp 70 genes. However, as only one Bgl I fragment was observed, the hsp 70 genes at 87A7 may be uniquely phased in which case those at 87C1 would exhibit at least two phases.

We can only speculate about the reason(s) why more than one phase occurs. It is conceivable that correct phasing of nucleosomes is crucial only in a small region containing control signals. In this region, for example at the 5'-end of the genes, nucleosomes may be phased uniquely, and the observed multiple phases would have to be attributed to slight differences between the sequences of the six genes downstream from this site. Alternatively, the signals might be exposed in a few equivalent phases of the nucleosomes.

What mediates the phasing of the nucleosomes? One possibility would be that it is a property of the histone octamers to associate with a given DNA sequence in one or a few preferential phases as it has been observed in reconstitution experiments of core histones with DNA of 203 and 144 base pairs (35). More attractive seems to us a mechanism in which the phase is not set in each nucleosome but only at certain sites from which it is propagated along the chromatin fiber. In this case, a nonhistone protein would recognize a signal in the DNA sequence that sets the phase. This protein might be the same as the one postulated to determine the limits of the protected domain in the repressed genes (Levy and Noll, submitted). The protected domain, which consists of 14 nucleosomes, extends from a site upstream from the 5'-end by about one nucleosome to a site close to the 3'-end of the hsp 70 gene (Levy and Noll, submitted).

From our previous study (Levy and Noll, submitted) and the measurements

of the nucleosome location in the hsp 70 genes reported here, the DNA ends of the core particles at the two limits of the protected domain may be determined more precisely. The right end is 30 to 50 base pairs downstream from the right-hand Sal site whereas the left boundary is about 250 or 170 base pairs upstream from the left Sal site (depending on the orientation of the nucleosome at the Bgl I site).

A signal that sets the phase or the limits of the protected domain by the interaction with a nonhistone protein is expected to be located in the flanking regions close to the nucleosome at the left or right end of the protected domain. Comparison with the DNA sequence (29, 30) in these regions reveals several intriguing features. At position 2204 of the hsp 70 gene at 87A7, a sequence of 10 base pairs starts which is repeated at position 2218-2227: 5'-ATGGGTTATA-3'. This sequence is not found at the 3'-end of the first of the hsp 70 genes at 87C1 (30; F. Karch, personal communication). It is striking that the last eight base pairs of this sequence are found as an inverted repeat with one mismatch (5'-TAGAACCC-3') between positions -246 and -239 which is close to the left end of the protected domain. Furthermore, the sequence 5'-AAAACAAAC-3' present at the left end of the protected domain between positions -236 and -228 occurs as inverted repeat at its right end between positions 2234 and 2242 in the hsp 70 gene at 87A7 or, with one mismatch (5'-GTTAGTTTT-3'), 45 base pairs further downstream in the first hsp 70 gene at 87C1 (30; F. Karch, personal communication). The seven base pairs in the middle (5'-AAACAAA-3') are found also as inverted repeat 27 base pairs downstream at the left end of the protected domain between positions -208 and -202. Although such sequences might represent phasing signals and/or determine the limits of the protected domain (Levy and Noll, submitted), more direct evidence for their significance is required.

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