Analysis of the DNase I-hypersensitive Site of a Developmentally Regulated 25-kDa Protein Gene of Sarcophaga peregrina*

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Change in chromatin structure of a developmentally regulated gene of Sarcophaga peregrina (flesh fly) during development was investigated. This gene (25-kDa protein gene) was specifically activated in the fat body, but not the hemocytes of larvae in the middle of the third instar. The mRNA level in the fat body decreased thereafter, reaching one-fifth of the maximum level in the late third instar to early pupal stage.

In the chromatin of fat body nuclei, a DNase I-hypersensitive site was found about 300 base pairs upstream from the transcription initiation site of the 25-kDa protein gene. This DNase I-hypersensitive site appeared before activation of the 25-kDa protein gene, and it was conserved until the late third instar, but disappeared in the early pupal stage. Since activity of the 25-kDa protein gene decreases significantly in the early pupal stage, it is likely that disappearance of this DNase I-hypersensitive site coincides with inactivation of the 25-kDa protein gene.

Transcription is a key step in selective gene expression in eukaryotic cells. The interactions of specific transcription factors with characteristic DNA structures in the vicinity of individual genes are thought to be essential for the regulation of eukaryotic gene expression (1, 2). It is, therefore, necessary to study both specific DNA structures and transcription factors to understand the mechanism by which gene expression is regulated.

DNase I-hypersensitive regions have been found in chromatin, and these are thought to be formed as the result of dynamic change in the structure of chromatin (3). Chromatin in the vicinity of actively transcribed genes or potentially active genes has been shown to have DNase I-hypersensitive sites (4), and in some cases, these sites were found to change during activation and inactivation of transcription (5, 6). Therefore, DNase I-hypersensitive sites could be good indicators of dynamic change in the structure of chromatin, which is thought to be related with activation or inactivation of transcription of specific genes, although no direct evidence for this has been obtained.

We previously reported the presence of two predominant mRNAs in the fat body of third-instar larvae of the flesh fly Sarcophaga peregrina (7). One of them was identified as mRNA for a storage protein that is a major larval hemolymph protein (8). The in vitro translation product of the other is a protein with a molecular mass of 25,000 daltons (25-kDa protein). We have cloned and sequenced the entire gene for 25-kDa protein (9). The function of the 25-kDa protein is not clear, but expression of the 25-kDa protein gene was found to be induced specifically in the fat body of middle third-instar larvae (10). This gene is a good model of developmentally regulated gene expression, since the timing of its expression is strictly regulated by the developmental stage.

This paper describes a DNase I-hypersensitive site in the vicinity of the 25-kDa protein gene. This site, which was upstream of the transcription initiation site of the 25-kDa protein gene, was found to change in a unique manner in relation to activation and inactivation of transcription of the 25-kDa protein gene. The relation between the existence of this DNase I-hypersensitive site and the level of transcription is discussed.

MATERIALS AND METHODS

Animals—The maintenance of S. peregrina was described elsewhere (11). Staging of larvae was done as follows. A mixture of adult males and females was kept at 27°C. Embryos grow in the abdomen of adult females and are laid on a piece of pork liver as first-instar larvae. Timing was started from this stage, which was taken as the hatching stage. Larvae were forced to pupate by drying their body wall in the late third-instar stage, as described by Ohtaki (11).

Isolation of Nuclei—Fat body tissues were dissected out of larvae or pupae in insect saline (130 mM NaCl, 5 mM KCl, and 1 mM CaCl2) at 0-4°C. Hemocytes were collected from hemolymph by centrifugation at 300 × g for 5 min and were washed well with insect saline. For collection of hemolymph, the anterior tip of larva was cut off with fine scissors and the hemolymph that exuded was dropped into a test tube kept at 0-4°C. Fresh tissues were used for isolation of nuclei. Nuclei were prepared by the method of Natori (12) with slight modifications. Fresh nuclei were used for analysis of DNase I-hypersensitive sites.

Analysis of DNase I-hypersensitive Sites—Nuclei were digested with DNase I essentially by the method of Wu et al. (3). Nuclei collected from about 0.1-0.3 g of wet tissue were incubated with various concentrations of DNase I in 250 μl of 15 mM Tris-HCl buffer, pH 7.4, containing 60 mM KCl, 15 mM NaCl, 0.5 mM dithiothreitol, 0.05 mM CaCl2, 3.0 mM MgCl2, and 0.25 mM sucrose for 3 min at 25°C. Digestion was terminated by adding final concentrations of 12.5 mM EDTA and 0.5% (w/v) SDS.1 DNA was then prepared from the lysed nuclei by successive extractions once with phenol:chloroform:isoamyl alcohol (50:1) (v/v) and twice with chloroform:isoamyl alcohol (50:1) (v/v) and was precipitated with ethanol. It was dissolved in 200 μl of 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, 100 mM NaCl, and 50 μg/ml RNase A and incubated at 37°C for 120 min. DNA was re-extracted from the reaction mixture, precipitated with ethanol, and dissolved in 100 μl of double-distilled water. Purified DNA (10-15 μg) was digested with HindIII (3-4 units/μg of DNA) in 100 μl of 100 mM Tris-HCl buffer, pH 7.6, containing 50 mM NaCl and 10 mM MgCl2 by incubation for 90 min at 37°C. The resulting digest was subjected to 0.7% agarose gel electrophoresis, transferred to a nitrocellulose filter paper (BA85, Schleicher & Schuell), and hybridized to a nick-translated DNA probe for the 25-kDa protein transcripts.

The abbreviations used are: SDS, sodium dodecyl sulfate; kb, kilobase pairs.

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kDa protein gene as described by Wahl et al. (13). The specific activity of the probe was usually 3-8 x 10^6 cpm/μg of DNA. After hybridization, the filter paper was washed three times with 2 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate) containing 0.1% SDS for 10 min at room temperature and once with 0.1 x SSC containing 0.5% SDS for 15 min at 68°C. The filter paper was then dried and autoradiographed.

Since digestion of Sarcophaga DNA with HindIII is known to give a DNA fragment of 4.0 kb containing the entire 25-kDa protein gene, change in the mobility of the DNA fragment hybridizable with DNA probe for the 25-kDa protein gene should give information on the DNase I-hypersensitive sites related to this gene.

**DNA Probe Used**—As described before (10), a Charon 4A vector containing the 11.2-kb insert of Sarcophaga DNA has been cloned. A 2.3-kb EcoRI-HindIII fragment containing the entire 25-kDa protein gene and 5'- and 3'-end flanking sequences was subcloned from the 11.2-kb insert, and its complete nucleotide sequence was determined (9). In this work, this 2.3-kb EcoRI-HindIII fragment was used throughout as a probe for detecting the 25-kDa protein gene and 25-kDa protein mRNA.

**Chemicals**—Restriction enzymes were purchased from Takara Shuzo Co. DNase I was from Worthington, and RNase A was from Sigma. [α-32P]dCTP (800 Ci/mmol) was from New England Nuclear.

**RESULTS**

**Change in Amount of mRNA for 25-kDa Protein during Development**—Previously, we showed that mRNA for 25-kDa protein (25-kDa protein mRNA) in the fat body increases dramatically in the middle of the third instar (10). For detailed determination of change in the amount of 25-kDa protein mRNA during larval development, we quantitated the mRNA by dot-blot hybridization using RNA prepared from animals synchronized at various developmental stages from the second instar to the early pupal stage.

As shown in Fig. 1, 25-kDa protein mRNA was not detected until 43 h after hatching, which is the early third instar. Thus, expression of this gene was almost negligible from before ecdisis to the early third instar. However, 67 h after hatching, which is in the middle of the third instar, the amount of 25-kDa protein mRNA increased dramatically to more than 600 times the background level. Then it decreased to one-fifth of this level in the late third-instar stage (124 h after hatching) and remained at this level until the early pupal stage. With pupal development, it decreased gradually, becoming undetectable in the late pupal stage, and was not present at a significant level in adult flies (data not shown). We recently demonstrated that accumulation of mRNA in the fat body directly reflects the activity of the 25-kDa protein gene in isolated fat body nuclei. Since the genomic organization of the 25-kDa protein gene is known not to change throughout development of Sarcophaga (10), we concluded from the present experiment that transcription of the 25-kDa protein gene is selectively activated in the middle of the third instar and that more than 80% of the gene is inactivated by the late third-instar stage. However, part of the 25-kDa protein gene remains active even after pupation, although the gene is gradually inactivated with development of adult structures.

**DNase I-hypersensitive Site of the 25-kDa Protein Gene**—Many genes that are active in transcription are known to have specific sites in flanking or transcribable regions that become sensitive to DNase I digestion on change in chromatin structure (4). These sites can be detected by dot-blot hybridization of DNA extracted from DNase I-treated nuclei with cloned DNA containing the respective gene as probe (3). Since expression of the 25-kDa protein gene clearly changes during development of Sarcophaga, we applied this method to the 25-kDa protein gene to determine whether the chromatin structure changes concomitant with gene activity in terms of DNase I hypersensitivity.

Nuclei were isolated from the fat body of middle third-instar larvae, in which transcription of the 25-kDa protein gene is very active, and the DNase I-hypersensitive site was analyzed as described under “Materials and Methods” with 2.3-kb EcoRI-HindIII DNA as a probe. As evident from Fig. 2a, when DNA was extracted from intact nuclei, a 4.0-kb band exclusively hybridized with the probe, indicating that the 25-kDa protein gene is located in the 4.0-kb HindIII-HindIII fragment. This band is intense and looks like a spot rather than a band, but this is simply due to overexposure of the x-ray film. The intensity of this band decreased, and a 2.3-kb band that was not detected in DNA from intact nuclei appeared with increase in the amount of DNase I used for digestion of the nuclei. The appearance of this 2.3-kb band was found to be due to the chromatin structure, since it was not detected when deproteinized DNA prepared from the fat body of middle third-instar larvae was treated in the same way, although the 4.0-kb band was clearly detectable (data not shown). Therefore, we concluded that the chromatin carrying the 4.0-kb HindIII-HindIII DNA fragment in the fat body nuclei of middle third-instar larvae contains at least a single DNase I-hypersensitive site. As reported before (9), this DNA fragment contains the entire 25-kDa protein gene with 2-kb 5'- and 1-kb 3'-flanking sequences. Judging from the size of DNA after digestion with DNase I, we concluded that the DNase I-hypersensitive site was located near the EcoRI site, which is about 300 base pairs upstream from the transcription initiation (cap) site (see Fig. 2b).

**Specificity of DNase I Hypersensitivity**—We know that the 25-kDa protein gene is specifically expressed in the fat body. If the DNase I-hypersensitive site is related to tissue-specific gene expression, it should also be tissue-specific. To test this possibility, we prepared nuclei from hemocytes of middle third-instar larvae and tested them for DNase I hypersensitivity. As shown in Fig. 3a, RNA prepared from hemocytes did not contain any appreciable 25-kDa protein mRNA, indicating that the 25-kDa protein gene is not transcribed in...
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FIG. 2. Analysis of DNase I-hypersensitive sites in chromatin of the fat body of middle third-instar larvae. a, DNase I-hypersensitive site in nuclei of the fat body of middle third-instar larvae was analyzed as described under "Materials and Methods." An autoradiogram of the washed filter paper after hybridization is shown. The concentrations of DNase I used for digestion of nuclei are indicated at the top of each lane in units/ml. HindIII-digested λ DNA was used as molecular size markers. The arrow shows the 2.3-kb band, which indicates the presence of the DNase I-hypersensitive site. b, location of the DNase I-hypersensitive site in the 25-kDa protein gene. The vertical arrow indicates the DNase I-hypersensitive site upstream of the 25-kDa protein gene. The thick horizontal arrow represents the 25-kDa protein gene with 5' and 3' orientation. E and H indicate the sites for endonucleases EcoRI and HindIII, respectively. The probe used for hybridization is shown by a line. The distance between the DNase I-hypersensitive site and the cap site of the 25-kDa protein gene was determined to be about 300 base pairs.

FIG. 3. Analysis of DNase I-hypersensitive sites in the chromatin of hemocytes. a, total cellular RNA was extracted from the fat body or the hemocytes of middle third-instar larvae and was used to quantitate the amount of 25-kDa protein mRNA as described for Fig. 1. F and H represent RNA from the fat body and hemocytes, respectively. b, nuclei were prepared from hemocytes of middle third-instar larvae and were used for analysis of DNase I-hypersensitive sites as described for Fig. 2a. HindIII-digested λgt was DNA was used as molecular size markers.

transcription of the 25-kDa protein gene was activated and persisted until the late third-instar stage, when expression of the 25-kDa protein gene decreased to one-fifth of the maximum level. It disappeared in the early pupal stage, when a detectable amount of 25-kDa protein mRNA was still present (Fig. 1). Animals in earlier developmental stages were not analyzed because of the technical difficulty of collecting sufficient fat body for analysis.

These results indicate that a DNase I-hypersensitive site is formed in fat body chromatin before induction of expression of the 25-kDa protein gene and that it is present during the time when the gene is active, but disappears in the early pupal stage when gene activity drops to one-fifth of that in the active stage before it is totally inactivated.

DISCUSSION

This paper describes the change in chromatin structure in the vicinity of the 25-kDa protein gene during development of S. peregrina. This gene is unique in the abundance of its mRNA in the fat body of third-instar larvae (10). Therefore, it is a good model for use in studies of developmental regulation of gene expression.

We found a DNase I-hypersensitive site about 300 base pairs upstream of the cap site of the 25-kDa protein gene in the fat body, but not in hemocytes. This site was detected until the late third instar when transcription had decreased to one-fifth of the maximum level. However, it finally disappeared in the early pupal stage, when a significant level of 25-kDa protein mRNA was still detectable.

There are several reports of appearance of a DNase I-hypersensitive site(s) before onset of transcription of a gene (5, 6, 14), but in this paper we clearly demonstrated that such a site disappears with inactivation of transcription of a gene. This DNase I-hypersensitive site was shown to be specific to chromatin of the fat body. It is not known at present whether this change in chromatin structure is responsible for selective expression of the 25-kDa protein gene. However, the possibility seems likely for the following two reasons. The DNase I-hypersensitive site is located in the proximal upstream region of the transcription site of the 25-kDa protein gene, which is a potential regulatory region of transcription (15). It is not
present in the chromatin of hemocytes in which the 25-kDa protein gene is not transcribed.

The relation between the DNase I-hypersensitive site and the level of transcription found in this study is quite unique. Therefore, it is tempting to speculate that transcription of the 25-kDa protein gene is regulated by the following mechanism. A DNase I-hypersensitive site is created in fat body chromatin by a specific transcription factor(s), such as that reported by Emerson and Felsenfeld (16), some time before the early third instar. This site represents a local perturbation of the structure of chromatin which may serve as a site of entry of a specific transcription factor(s) and/or RNA polymerase II (17). Transcription of the 25-kDa protein gene may not start at this stage, because of absence of a specific transcription factor(s) that directly induces transcription of the 25-kDa protein gene interacting with RNA polymerase II and/or characteristic DNA structures, including the DNase I-hypersensitive site. Transcription is initiated in the middle of the third instar when this specific factor(s) is ready for use. In the late third instar, transcription of the 25-kDa protein gene decreases to one-fifth of the maximum level, although the DNase I-hypersensitive site remains intact. Probably, at this stage, the activity of the transcription factor(s) itself is reduced in some way, causing a change in chromatin structure, resulting in disappearance of the DNase I-hypersensitive site in the early pupal stage. Disappearance of the DNase I-hypersensitive site may be responsible for complete cessation of transcription of the 25-kDa protein gene in this later stage.

In order to understand the mechanisms of developmental regulation of activation and inactivation and efficient transcription of the 25-kDa protein gene of Sarcophaga, it is essential to analyze the mechanism of stage-specific and tissue-specific appearance and disappearance of the DNase I-hypersensitive site. For this, many more experiments are needed, including determination of the DNA sequence of the DNase I-hypersensitive region and identification of the protein(s) interacting with this DNA sequence. The present results should provide a clue to this problem.