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Detection of the Specific DNA-binding Proteins for the Aphid rRNA

진딧물 rRNA 유전장에 특이적으로 결합하는 단백질 탐색

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ABSTRACT A whole body extract (WBE), a crude nuclear fraction, of aphids was prepared and used to identify the proteins which bound specifically to 5'-upstream regions of the transcription initiation site of the aphid ribosomal RNA gene (rDNA). While DNA fragment C(-263/-195) was bound by only one specific 53 kDa protein, two DNA fragments, A(-194/23) and B(-393/-264), were commonly bound by three proteins (52 kDa, 50 kDa and 40 kDa) It was also revealed that the formation of the DNA-protein complex requires a cation.

KEY WORDS Aphids, *Acythosiphon pisum* (Harris), Whole body extract, Ribosomal RNA gene, Transcription initiation site

초 록 정확한 *in vitro* 전사가 일어날 수 있는 진딧물의 세포추출액을 제조하였다. 전사를 직접 조절할 수 있는 단백질 인자를 규명하기 위하여 전사개시점과 그의 상류에 결합하는 DNA 결합단백질을 탐색했다. 전사게시점을 포함하는 단편 A(-194/23)에는 52 kDa, 50 kDa, 40 kDa의 단백질들이 결합했으며 전사개시점 상류의 DNA 단편 B(-393/-263)에는 52 kDa, 50 kDa, 40 kDa의 단백질들이 결합한 반면 DNA 단편 C(-263/-195)는 53 kDa단백질만이 결합했다. 그리고 이들 DNA 결합단백질들의 DNA 결합 활성에는 양이온이 요구되었다.

검색어 진딧물, *Acyrthosiphon pisum* (Harris), 세포추출액, 라이보좀 RNA 유전자, 전자개시점

In all eukaryotes, rDNA are organized as tandem repeats of a single transcriptional unit containing the rRNA coding sequences separated by intergenic transcribed spacer (IGS) and external transcribed spacer (ETS). Transcription of rDNA by RNA polymerase I starts in the ETS, proceeds through the rRNA coding sequences and terminates in the following IGS. The pre-rRNA is then modified by multiple specific RNA processing steps to generate the mature cytoplasmic rRNAs (5.8S, 18S and 28S RNA).

Studies on *in vitro* and *in vivo* transcription of the eukaryotic rDNA have revealed that *trans*-acting elements are required to transcript exactly from transcription initiation site. There are several kinds of species-specific *trans*-acting elements such as upstream DNA binding proteins, RNA polymerase I promoter selectivity factors (SL 1), upstream binding protein (UBF) and RNA polymerase I associated proteins (Bell *et al.* 1988, Comai *et al.* 1992, Echeverria *et al.* 1994, Reeder 1990, Sollner-Webb & Mougey 1991). In view of the fact that the eukaryotic rDNA is transcribed species-specifically by RNA polymerase I, it is reasoned that at least one of the these factors is species-specific (Grummt *et al.* 1982). While these proteins are thought to bind to upstream control elements (UCE) of the promoters and to regulate the expression of eukaryotic rDNA, the molecular mechanisms of the modulation of transcription by these *trans*-acting factors have not

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been fully elucidated. Recently, various protein factors controlling rDNA transcription in a species-specific manner have been identified and the corresponding cDNAs have been cloned from several species.

To understand the molecular mechanisms of expression and regulation of eukaryotic rDNA in general, we used aphid rDNA as a model because of its unique molecular properties (Ishikawa 1975, Kwon *et al.* 1991, Kwon & Ishikawa 1992a, b). It was difficult until recently that the preparation of a crude nuclear containing active proteins from the whole body of aphids which contain several kinds of free-living bacteria in the midgut and intracellular symbionts (Harada & Ishikawa 1993). We here report the first successful preparation and use a whole body extract from aphids for DNA-binding experiments.

MATERIALS AND METHODS

A long-established parthenogenetic clone of the pea aphid, *Acyrthosiphon pisum* (Harris) was reared on young bread-bean plants, *Vicia faba L.* at 15°C in a short-day regime with a 12h photoperiod.

The DNA fragments (A, B and C) used in this study were prepared from the P^Ap^R869 plasmid, which contains the 5'-upstream region of aphid rDNA (Kwon & Ishikawa 1992a). First, the plasmid p^Ap^R869 was digested with SacI-SmaI. The resulting fragments were digested with Sau3AI, and they were subsequently ligated into the BamHI sites of the plasmid Bluescript SK^+ to obtain fragments A (0.22 kb), B (0.13 kb) and C (0.07 kb). Each fragment was labeled with [^{32}p]dCTP using the Klenow fragment of DNA polymerase I (Kwon & Ishikawa 1992 a).

Aphid tissues were homogenized manually in liquid nitrogen in a motor and pestle. All the subsequent procedures were performed at 4°C. The homogenate was mixed with two volumes of extraction buffer (100 mM Hepes-HOH (7.9)/245 mM KCl/5 mM EGTA/1 mM EDTA/2.5 mM DTT) containing protease inhibitors (0.2 mM PMSF, 10 mM Benzamidine, 25 μg/ml TLCK, 3.5 μg/ml Pepstatin A, 5 μg/ml Leupeptin, 10 μg/ml Apotinin, 10 μg/ml Chymoststin). The tissue suspension was briefly mixed by pipetting up and down and then centrifuged at 100,000×g for 2h. The supernatant was dialyzed against 50 volumes of 20 mM Hepes-KOH (7.9)/50 mM KCl/5 mM EGTA/0.05 mM EDTA/2.5 mM DTT/20% glycerol/0.2 mM PMSF/0.5 μg/ml Leupeptin for 4h. After fractionation through Sepharose CL 6B (Pharmacia), the peak protein fractions pooled and stored at −80°C until the following experiments (Schultz *et al.* 1991).

Protein concentrations were measured by the method of Bradford (Bradford 1976). For DNA-protein binding, DNA fragments were suspended in WBE and binding buffer (12 mM Hepes-KOH (7.9), 12% glycerol, 60 mM KCl, 0.1 mM EDTA, 0.6 mM DTT, 5 mM $MgCl_2$). As a DNA competitor, heparin solution was added to a final concentration of 0.2 μg/μl. The binding reaction was carried out on ice for 30 min and the resulting reaction mixture was directly loaded on a 5% polyacrylamide gel (Fried & Crothers 1981, Lee & Schwartz 1992).

The whole body extract of pea aphids was mixed with loading buffer (2.5% SDS, 5% mercaptoethanol, 20% glycerol, 87.5 mM Tris-HCl (6.8)), and denatured at 60°C for 2 min. The proteins were separated on a 5% SDS-PAGE and transferred to nitrocellulose filter in transfer buffer (25 mM Tris-HCl (6.8), 192 mM glycine, 20% methanol and 0.01% SDS). The filter was soaked in binding buffer 1 (10 mM Tris-HCl (7.5), 1 mM EDTA, 50 mM NaCl, and 3 mM $MgCl_2$) at room temperature for 40 min. The filter was transferred to binding buffer 2 (10 mM Tris-HCl (7.5), 1 mM EDTA, 50 mM NaCl, 3 mM $MgCl_2$, 0.02% BSA, 0.02% Ficoll, and 1 mM DTT) and incubated with a DNA competitor at room temperature for 20 min. Subsequently, the labeled DNA fragments (A, B and C) were added to the reaction buffer and incubated for 40 min at room temperature. After the binding reaction, the filter was washed three times in binding buffer 1 for 10 min, air-dried and autoradiographed (Schmitz *et al.* 1989).

RESULTS AND DISCUSSION

Ribosomal transcription in many eukaryotes depends on a complex array of repeated enhancers, du-

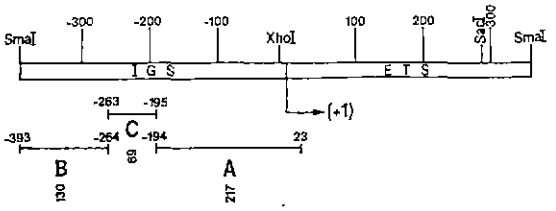

Fig. 1. Schematic illustration of the 5′-upstream region of the aphid rDNA. The location of fragments A, B and C, used as probes, was shown. Positions were numbered relative to the site of transcription initiation (+1).

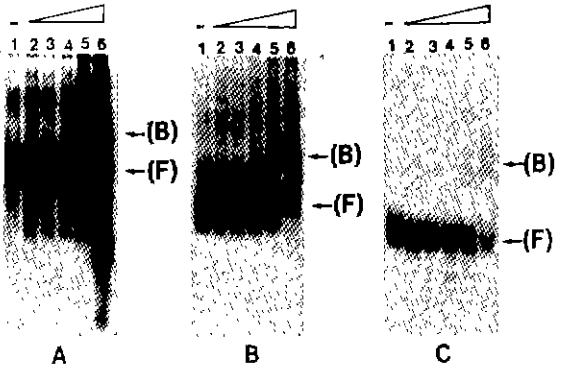

Fig. 2. Gel mobility shift assay. Panels A, B and C correspond to experiments with fragments A, B and C, *respectively. In the three panels,* lanes 1 *represent* the control without DNA competitor and lanes 2 to 6, with 0.1, 0.3, 1, 3 and 10 μl of WBE, respectively. DNA-protein complexes (B) and free DNA (F) were indicated by arrorws.

plicated promoters, and terminators. Most of these rDNA elements (*cis*-acting elements) are recognized by several protein factors (*trans*-acting elements) required for efficient promoter activation.

We have previously reported nucleotide sequences of 18S, external transcribed spacer (ETS) and intergenic transcribed spacer (IGS) of the aphid rDNA (Kwon *et al.* 1991, Kwon & Ishikawa 1992a, b). to identify *trans*-acting elements, first of all we looked for the proteins that could bind specifically to the upstream region of transcription initiation site of the aphid rDNA. For this purpose, we subcloned the DNA fragment encompassing the IGS region from the plasmid p^Ap^R869 (Kwon & Ishikawa 1992 a). This DNA fragment was digested with Sau3AI, and the three resulting fragments (A, B and C as shown in Fig. 1) were examined for following experiments.

In order to get evidences that the three DNA fragments (A, B and C) are bound by specific proteins in WBE, gel mobility shift experiment was performed (Echeverria *et al.* 1992). In Fig. 2, panels A, B and C correspond to fragment A, B and C used in this experiments, respectively. In lanes 2-6, the reaction mixture contained WBE, labeled DNA fragments, DNA competitor in binding buffer. The samples, in lane 1 contained all these components but the DNA competitor. On lanes 5 and 6 of panels A, B and C, the positions of migration of the DNA-protein complex (B) and free DNA (F) were detected, which were indicated by arrows. The band B became more distinct with increasing amount of WBE (lanes 2 to 6). This result reveal that there are proteins in WBE that bind specifically to the 5′-upstream regions of the aphid rDNA and slowly moved bands indicate the affection of extract-dependent retardation.

To confirm that the slower migrating bands (B in Fig. 2) actually represent DNA-protein complexes, the protease sensitivity of these bands was examined by the gel mobility shift assay with trypsin. After the reaction of the DNA fragment with WBE, trypsin was added to digest the proteins which had bound to the DNA fragment. The result was shown in Fig. 3. When the samples were treated with trypsin (1 μg/ml) (lanes 2 in each panel) the band B in each lane disappeared, suggesting that this band represents a DNA-protein complex.

Since the formation of DNA-protein complex was markedly reduced in the presence of 0.2 mM EDTA in the reaction mixture for the gel mobility shift assay (data not shown), effects of various cations on the complex formation with the fragment C were examined. As shown in Fig. 4, all the cations tested increased the complex formation, but Mg^{2+} (lane 3) and Zn^{2+} (lane 4) were more effective.

It is well known that the concentration of a cation in the vicinity of the *cis*-acting element may be important for the formation of DNA-protein complex. We examined the effect of the concentration of

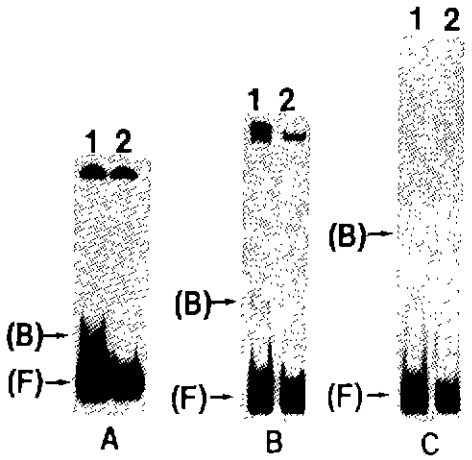

Fig. 3. Effect of trypsin on DNA-protein complex. Panels A, B and C correspond to experiments with fragments A, B and C, respectively. In the three panels, experiments in lanes 1 were undertaken under the same conditions as in the gel mobility shift assay. In lanes 2 trypsin (1 μg/ml) was added after the formation of DNA-protein complex. DNA-protein complex (B) and free DNA (F) were indicated by arrows

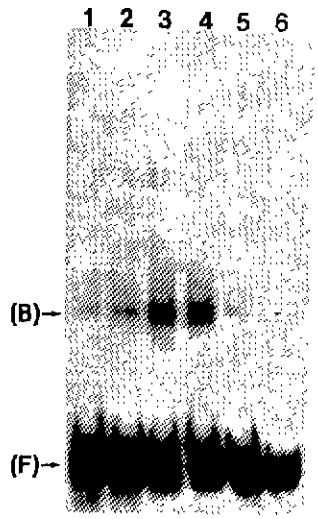

Fig. 4. DNA-protein complex formation in the presence of various cations. The WBE was first treated with 0.2 mM EDTA on ice for 15 min, then a final concentration of 0.3 mM of the indicated cation was added, and gel mobility shift assay was performed with fragment C. Lanes 1 to 6, with $CaCl_2$, $MnCl_2$, $ZnCl_2$, $CoCl_2$ and $BaCl_2$, in this order. The positions of migration of the DNA-protein complex (B) and free DNA (F) were indicated by arrows.

Fig. 5. Effect of $MgCl_2$ on DNA-protein complex formation. Gel mobility shift assay was performed with fragment C in the presence of increasing amount of $MgCl_2$. Lane 1, with 0.3 mM EDTA alone; Lane 2 to 4, first added 0.3 mM EDTA, and then 0.01, 0.5 and 5 mM of $MgCl_2$ were added, respectively. The positions of migration of the DNA-protein complex (B) and free DNA (F) were indicated by arrows.

$MgCl_2$ in the reaction mixture on the DNA-protein complex formation. As shown in Fig. 5, gel-mobility shift assay was performed with fragment C in the presence of increasing amount of $MgCl_2$ from lane 2 to lane 4. Lane 1 represents a control experiment with only EDTA, without $MgCl_2$, where no band was detected. The DNA-protein complex formation was enhanced significantly in the presence of 0.5 mM $MgCl_2$ (lane 3). As shown in lane 4, $MgCl_2$ at a concentration higher than 0.5 mM $MgCl_2$ was rather inhibitory, suggesting that the moderate concentration of a cation is required for a DNA-protein complex formation.

To estimate the number of subunit and molecular weight of these proteins binding to the three different DNA fragments, South-Western experiments (Maeda *et al.* 1992) were undertaken. As shown in Fig. 6, panels A, B and C represent DNA fragments A, B and C, respectively. Three proteins with molecular mass of 52 kDa, 50 kDa and 40 kDa were found bound in common with fragments A and B.

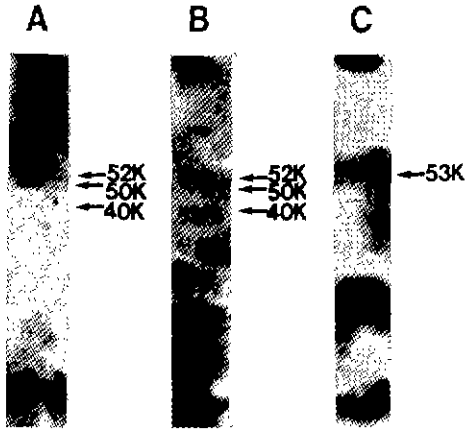

Fig. 6. South-Western experiments. The WBE were separated by electrophoresis, transferred to nitrocellulose filters, and incubated with the labeled DNA fragments (A, B and C) as described in the methods In the three panels, arrows indicate the positions of DNA-protein complexes and estimated molecular weight of bound proteins

In contrast, with the fragment C, only a 53 kDa protein was found bound. In terms of the same molecular mass and most aspect of effective protein homeostasis in a cell, we can not rule out the possibility that these proteins, 52 kDa, 50 kDa and 40 kDa, bound with fragment A and B are the same.

Recent studies have indicated that multiple-*cis*-acting elements and *trans*-acting factors including DNA-binding proteins are involved in the expression of rDNA by RNA polymerase I (Bell *et al.* 1988, Comai *et al.* 1992, Echeverria *et al.* 1992, Reeder 1990, Sollner-Webb & Mougey 1991). Since IGS of the aphid rDNA contains unique structure as described (Kwon 1992a), it is likely that the four proteins found in the present study bind to the aphid IGS which may take part in the species-specific regulation of expression of the aphid rDNA Further experiments, DNAse I footprinting, and methylation interference analysis, will be carried out to identify these sequences and secondary structures should be recognized by four different proteins (53 kDa, 52 kDa, 50 kDa and 40 kDa) which may be playing a pivotal role in pre-rRNA processing. Also whether they are specific RNA polymerase I transcription factors or play a more general role in genome expression retains to be elucidated (Bazett-Jones *et al.* 1994).

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