Development 114, 481-491 (1992)
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Identification and characterization of *Drosophila* female germ line transcriptional control elements

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Summary

The highly organized structure of the *Drosophila* ovary makes it an ideal system for studying mechanisms of differential gene expression. Here we report the identification of a 171 bp sequence from the 5′ end of the *hsp26* gene that functions as a female germ-line-specific transcriptional regulator when linked in two copies to a basal promoter. The regulator is active only in non-dividing cells of the germ line, i.e., only in nurse cells and oocytes. It is not active in any examined tissue or cell type outside of the female germ line. Copper nuclease footprinting studies show that the germ line regulator contains two binding sites for each of two different ovarian nuclear factors. Point mutations in the DNA target sites of either nuclear factor abolish *in vitro* binding and *in vivo* transcriptional activity, indicating that each factor is a positive activator of nurse cell/oocyte transcription. The two factors may represent different classes of activator proteins, since an increase in the copy number of one factor's DNA target site cannot compensate for a decrease in the copy number of the other factor's target site.

Key words: *Drosophila* oogenesis, regulatory elements, DNA-binding, transcription factor, transcription, P-element transformation.

Introduction

A fundamental goal of molecular biology is to understand how genes are switched on and off in response to specific developmental stimuli. *Drosophila* oogenesis is an ideal system for studying such events, since the lineage of each ovarian cell type can be determined by a number of morphological and positional criteria. Further, genes have been identified that are expressed in defined cellular subsets of the ovary. In the ovary, therefore, it is possible to identify a cell that expresses a particular gene as well as its non-expressing sister and/or mother cells.

The *Drosophila* germ lineage contains a single branch point. Founder ooblasts (also called cystoblasts) divide with incomplete cytokinesis four times to produce an interconnected cluster of 16 cystocytes, one of which becomes the oocyte, while the rest become nurse cells (Brown and King, 1964; King, 1970; and see Fig. 1). The first visible signs of nurse cell versus oocyte differentiation coincide with the encapsidation of the 16-cystocyte cluster by a monolayer of somatic follicle cells to give the egg chamber (Brown and King, 1964; King, 1970; Mahowald and Kambysellis, 1980; Margaritis et al., 1980). Subsequent steps of egg chamber maturation have been divided into 14 morphologically identifiable stages (King et al., 1956; King, 1970).

In this paper, we focus on the regulated transcription of the *hsp26* gene. *hsp26* is representative of a large class of genes whose transcription is restricted to non-dividing germ cells, that is, to nurse cells and oocytes (Zimmerman et al., 1983; Glaser et al., 1986; also see Grossniklaus et al., 1989; Fasano and Kerridge, 1988). Other genes in this class include *dorsal* and *bicoid*, which function critically in embryonic pattern formation (Frigerio et al., 1986; Steward et al., 1985; Nüsslein-Volhard and Roth, 1989). We have focused on *hsp26* transcription for the following reasons. (1) The gene is expressed to high levels in nurse cells. Thus, its transcriptional control elements should be useful in experiments aimed at over-expressing or ectopically expressing cloned genes in the female germ line. (2) The approximate location of its *cis*-acting regulatory elements was determined in previous studies (Cohen and Meselson, 1985; Glaser et al., 1986). (3) The gene is not normally expressed in any other cell type of the adult female (Cohen and Meselson, 1985; Glaser et al., 1986). Therefore, some of its regulatory factors are probably needed exclusively during oogenesis, which would make them amenable to genetic analysis.

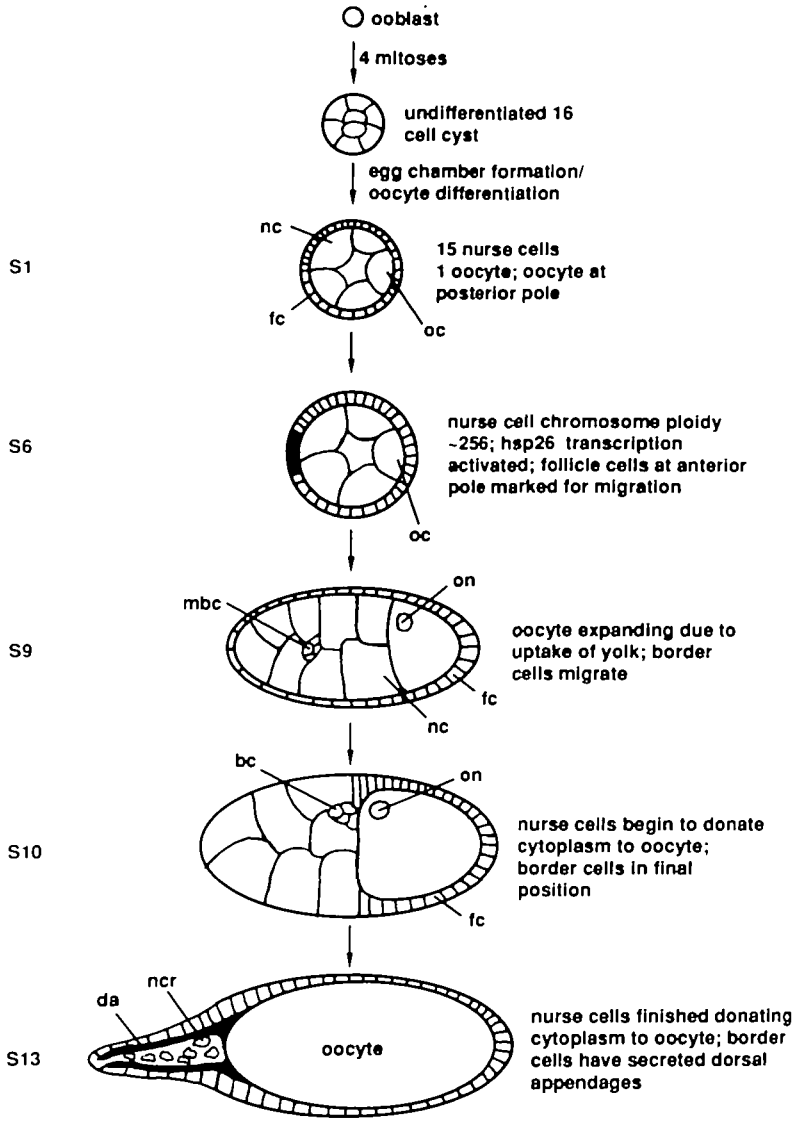


Fig. 1. Schematic diagram of *Drosophila* oogenesis (adapted from King, 1970). Oogenesis begins when a stem cell, located at the anterior tip of the ovary (not shown), divides. One of the stem cell's daughters, the ooblast, divides four times with incomplete cytokinesis to give a cluster of 16 cells connected to one another by cytoplasmic bridges. One of these cells migrates to the posterior pole of the cluster and becomes the oocyte, while the other 15 cells adopt the nurse cell fate. Coincident with visible signs of nurse cell/oocyte commitment, the 16-cell cluster is surrounded by a monolayer of mesodermally derived follicle cells to give a stage 1 egg chamber. The maturation of the oocyte and nurse cells within the egg chamber has been divided into 14 morphologically identifiable stages, several of which are illustrated in the diagram. S, stage of oogenesis; nc, nurse cell; fc, follicle cell; oc, oocyte; on, oocyte nucleus; mbc, migrating border cell; bc, border cell; ncr, nurse cell remnant; da, dorsal appendage.

In previous work, we showed that the transcription of the *hsp26* gene in the female germ line is dependent on information contained within the 171 bp sequence −543 to −373, where +1 corresponds to the transcription start site (Cohen and Meselson, 1985). Here, we show that two copies, but not one copy, of the 171 bp sequence can activate a basal promoter in nurse cells and, to a lesser extent, oocytes. The requirement for two copies of the 171 bp sequence indicates that the 171 bp sequence contains at least one copy of all of the control elements necessary for nurse cell/oocyte transcription, but that at least one such element must be present in multiple copies. Consistent with this idea, we find that the 171 bp sequence contains one binding site for each of two different ovarian-enriched nuclear proteins, which also bind to other regions of the *hsp26* gene. Each ovarian protein appears to provide a different essential function in the activation of transcription, and/or to be present in limiting quantities, since mutations that eliminate the binding of either protein to the 171 bp sequence also eliminate *in vivo* transcriptional activity, even when the number of target sites for the other protein is increased. Binding sites for these proteins are found in the promoter regions of *bicoid* and *hunchback*, suggesting that they mediate the transcription of a variety of genes in the female germ line.

Materials and methods

P element-mediated transformations

Embryos were prepared for microinjections as previously described (Cohen and Meselson, 1985). Test DNAs (diagrammed in Fig. 2) were cloned into the Carnegie20 transformation vector (Rubin and Spradling, 1983) and injected at a concentration of ~300 μg/ml. Transposase was provided by the helper plasmid, p13πwc (Cohen and Meselson, 1985), which was coinjected at a concentration of ~75 μg/ml. Between 3 and 12 transformed lines were generated and analyzed for each construct. Transformants were maintained as homozygous or balanced heterozygous stocks.

Gene constructs

The *lacZ* reporter gene was constructed by linking the −127 to +38 sequence of the *D. melanogaster Sgs-3* gene in-frame to the 5′ end of a *lacZ-Sgs-8* fragment. The *Sgs-3* sequence, isolated from plasmid Dm2023 (kindly provided by E. Meyerowitz), lacks the *Sgs-3* salivary gland enhancer, but contains promoter elements sufficient for a low level of salivary gland expression (Meyerowitz et al., 1987). The *lacZ-Sgs-8* fragment, isolated from plasmid ploxba (kindly provided by V. Corbin), includes the entire *E. coli lacZ* protein coding sequence, exclusive of residues 1-9, and 268 bp of DNA from the 3′ end of the *Drosophila Sgs-8* gene, which includes a functional poly(A) addition site (Garfinkel et al., 1983). The *lacZ* reporter thus produces a tripartite mRNA of structure *Sgs-3-lacZ-Sgs8*. The translation start and stop sites are provided by the *Sgs-3* and *lacZ* segments, respectively. All but the first two residues of the protein product derive from the *lacZ* segment. This *lacZ* reporter was cloned as an *Xho*I-*Sal*I fragment into the *Sal*I site of a modified Carnegie20 vector and is oriented in the anti-parallel direction relative to the ry^+ marker gene. The modified Carnegie20 vector differs from Carnegie20 in that the *Hpa*I site of the latter was converted to an *Xba*I site.

hsp26 test DNAs (diagrammed in Fig. 2) were inserted with the help of synthetic linkers at an *Xba*I site, which lies 146 bp upstream of the transcription start site of the *lacZ* reporter gene, and/or at a *Sal*I site, which lies 6.5 kb downstream of the transcription start site of this reporter gene (Fig. 2). *hsp26* test DNAs derive from plasmids p26(−743 to −373) and p26(−543 to −373), which consist of the *hsp26* sequence −743 to −373 and −543 to −373, respectively, where +1 corresponds to the *hsp26* mRNA start site, cloned into the *Asp*718-*Xba*I sites of the Bluescript plasmid vector (Stratagene, Inc.). The 5′ ends of the −743 to −373 and −543 to −373 sequences are defined by synthetic *Asp*718 restriction sites, which were added to the *hsp26* sequences following *Bal*31 digestion. The 3′ ends of both clones are defined by the same naturally occurring *Xba*I site. The complete sequence of the −743 to −373 domain is available upon request. The CAACAA and AATAA element mutations were generated by ligating appropriate 3′ and 5′ *Bal*31 deletion fragments of the −543 to −373 sequence to each other. The CAACAA element mutation is a triple point mutation, in which the wild-type element (GACAACAACTAC) is converted to the sequence GACAACGGCCAC. The AATAA element mutation is a heptuple point mutation in which the wild-type element (GAATAAAAAAAACT) is converted to the sequence GGCTAGAGATCTCT. In addition, this mutated element is flanked by a 4 bp (i.e., ~one half turn of B form DNA) insertion. Although alterations in the spacing between certain regulatory elements may interfere with their ability to interact with each other (Ptashne, 1986; Takahashi et al., 1986; Cohen and Meselson, 1988), the level of interference (when assaying transcription) is generally small (<5-fold). Significantly, the AATAA element mutation decreases the transcriptional activity of the 171 bp sequence by more than 25-fold. (With two copies of the wild-type 171 bp sequence, β-gal staining activity is detectable in nurse cells and oocytes within ~5 minutes of the addition of the color substrate, while with two copies of the 171 bp sequence specifically mutated at AATAA, no β-gal staining activity is detected after 24 hours of staining). We also note that an 18 bp insertion immediately downstream of the CAACAA element (i.e., between the CAACAA and AATAA elements) has no affect on the transcriptional activity of the 171 bp sequences (data not shown). Insertions of multiple copies of a particular DNA fragment were accomplished by ligating at high insert concentration. Further details regarding plasmid constructions will be furnished upon request.

Histochemistry

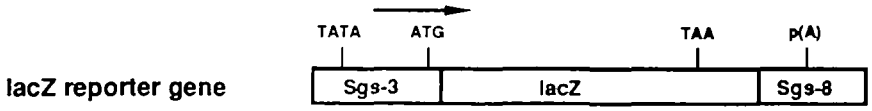
Desired tissues were dissected in PBS* (phosphate-buffered saline containing 2 mM $MgCl_2$, 2 mM spermidine, 0.02% sodium deoxycholate and 0.02% Nonidet P-40) and fixed for 10 min in a 4% paraformaldehyde solution freshly made in PBS*. Fixed tissues were rinsed with PBS* and then placed in staining buffer (PBS containing 0.2% X-gal, 1.1 mM $K_3Fe(CN)_6$ and 1.1 mM $K_4Fe(CN)_6$ and 2 mM $MgCl_2$). Staining reactions were carried out at 37°C in dark, moistened chambers, until the desired intensity was obtained. For constructs containing two or more intact copies of the 171 bp sequence (e.g., *2x171-Z*) this was typically ~30 min. For other constructs, such as *171-Z-3x171* and *171/2xmutCAACAA-Z*, the staining reaction was carried out for several hours. Stained tissues were mounted in Polyaquamount (Polysciences, Inc.) and photographed with Nomarski optics.

Band shift assays

DNA-binding reactions were carried out on ice in a volume of 20 μl. Reactions consisted of 4 mM Tris (pH 7.5), 0.5 mM DTT, 5% glycerol, 150 mM NaCl, 2 μg poly[d(IC)], 1 mM EDTA, 0-500 ng unlabeled competitor DNA, 1-2 ng (~50 femtomoles) ^{32}P-end-labeled substrate DNA, and ~5 μg (1 μl) nuclear extract (see below for extract preparation). After 25 minutes on ice, loading dye was added and the mixture was electrophoresed through a 0.5 × TBE/5% glycerol/4% polyacrylamide gel at 4°C. The bromophenol blue was run 3/4 the length of the gel. The gel was removed from the plates, wrapped in plastic wrap, and exposed to X-ray film at −80°C overnight with an intensifying screen.

Copper nuclease footprinting

The DNA-binding reaction described above was scaled up 15-fold and subjected to gel electrophoresis as above. After electrophoresis, one plate was removed and the gel still attached to one plate was submerged in 200 ml Tris-HCl (pH 8.0). The copper nuclease reaction was started by adding 20 ml of freshly prepared nuclease solution (0.45 mM $CuSO_4$ and 2 mM 1,10-orthophenanthroline) and 20 ml of freshly prepared catalyst (100 μl mercaptopropionic acid in 19.9 ml water) as described (Kuwabara and Sigman, 1987). The copper nuclease solution was made by adding 1 ml of 9 mM $CuSO_4$ (prepared in water) to 1 ml 40 mM 1,10-orthophenanthroline solution (prepared in 100% ethanol). After about 1 minute this solution turns blue. It is then diluted with 18 ml of water. The gel was incubated with the diluted nuclease solution for 15 minutes at room temperature, without shaking. The nuclease reaction was stopped by adding 15 ml of 50 mM 2,9-dimethyl-1,10-orthophenanthroline (made in ethanol) and waiting 2 minutes. The gel was then rinsed thoroughly with water and exposed to X-ray film overnight at 4°C. The free and protein-bound DNAs were cut out of the gel and the gel pieces diced. The DNA was eluted overnight on a rotating wheel at 37°C in 716 μl Maxam-Gilbert elution buffer (0.5 M ammonium acetate, 0.1 mM EDTA, 0.1% SDS) containing 80 μl methanol, 4 μl Proteinase K (10 mg/ml), and 1 μl yeast RNA (5 mg/ml) (Treisman, 1986). Acrylamide was removed from the elution buffer by passage through a 0.45 μm cellulose acetate filter. The flow-through was extracted once with phenol/chloroform and precipitated with 2.75 volumes ethanol. The DNA was reprecipitated twice, once with 0.5 mM spermine (Treisman, 1986) and once with ethanol to remove impurities. The final DNA pellet was washed once in 70% ethanol, redissolved in sequencing gel sample buffer



Inserts (hsp26 and synthetic DNA fragments)	insert # (location)	fusion name	nurse cell/oocyte expression
-746 -544 -543 -373	1 (5')	203/171-Z	++
	1 (5', rev)	rev203/171-Z	+++
-543 -373	1 (5')	171-Z	-
	1 (5', rev)	rev171-Z	-
	2 (5')	2x171-Z	+++++
	4 (3')	Z-3x171	-
	1 (5') & 3 (3')	171-Z-3x171	++
-457 -373	4 (5')	4x85-Z	-
-543 -373	3 (5')	mutCAACAA-Z	-
-543 -444	2 (5')	2x100-Z	-*
	3 (5')	3x100-Z	-*
-543 -373	4 (5')	mutAATAA-Z	-
-457 -373 -543 -373	1 (5')	85/171-Z	+++
-543 -373 -543 -373 2x( )	1 (5')	171/2xmutAATAA-Z	-
-543 -373 -543 -373 2x( )	1 (5')	171/2xmutCAACAA-Z	++

(Maniatis et al., 1982), heat-denatured and applied to a 6% or 8% sequencing gel.

Preparation of ovarian and non-ovarian nuclear extracts

All of the following steps were carried out on ice. About 1500 ovaries (~1 ml loosely-packed volume) were dissected over a two-day period from healthy, egg-laying, three to five day old Oregon R females in M3 media (Shields and Sang, 1977). The ovaries were rinsed twice with MTBS (135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM Na_2HPO_4, and 2.5 mM Tris-HCl, pH 7.5) to remove food particles. Egg chambers were liberated from the ovaries by gently douncing in a 1.5 ml tube with a loose-fitting plastic plunger. Care was taken not to break the cells at this point. Egg chambers were collected by centrifugation (2200 rev./minute, 7 minutes) and washed with 5 ml MTBS. Washed egg chambers were resuspended in two volumes of solution I (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol and 0.5 mM PMSF), transferred to a 1.5 ml tube, and dounced thoroughly. Nuclei and other cell debris were collected by centrifugation at 4000 rev./minute for 4 minutes. A small

Fig. 2. Mapping regulatory elements responsible for nurse cell and oocyte transcription. The *lacZ* reporter gene is diagrammed at the top of the figure (not to scale). This reporter is not expressed in any examined adult, larval or embryonic tissue, except salivary glands in which it is expressed to a low level (data not shown). Test DNAs are diagrammed below the *lacZ* reporter. Each test DNA derives from *hsp26* genomic DNA sequences. The numbers above the 5′ and 3′ ends of such fragments denote their genomic position with respect to the *hsp26* start of transcription. The positions of the GAATAAAAAAAACT and GACAACAACTAC regulatory elements (abbreviated AATAA and CAACAA, respectively) are indicated by the filled and stippled bars, respectively. The nucleotide positions of these elements are: AATAA, −436 to −423, and CAACAA, −512 to −501. The crosses drawn over the AATAA and CAACAA bars of some fragments indicate that these elements are mutated (see Materials and methods for sequence). The test DNAs were inserted in single and/or multiple copies, 5′ and/or 3′ to the *lacZ* reporter (see insert # and location) to give a total of 15 fusion constructs. "rev" indicates that the test DNA was inserted in the reverse orientation. The expression of each fusion construct was analyzed in at least three and in as many as 12 independently transformed lines using a histochemical assay for β-gal enzyme activity (Materials and methods). The results of such analyses are summarized in the right-hand column; blue reaction products detectable within 30 minutes (+++++), 30 minutes to 2 hours (+++), 2 to 4 hours (++), and no signal after overnight staining (−). Apart from the *171-Z-3x171* construct which was expressed in just 2 of the 8 lines analyzed, and the *171/2xmutCAACAA-Z* construct, which was expressed to variable levels (see text), each construct gave a very consistent staining pattern from line to line. The asterisk indicates expression in border cells (see text). Apart from this border cell expression, none of the test DNAs activated the *lacZ* reporter in any examined cell type or tissue other than nurse cells and oocytes.

volume of the pellet was examined to determine its purity. If greater than 10% of the pellet consisted of unbroken cells, the homogenization step was repeated. The nuclei were eventually resuspended in 2 volumes of solution II (10 mM HEPES, pH 7.9, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM PMSF and 5% glycerol) and, following the addition of 5 M NaCl to a final concentration of 0.35 M, incubated on ice for 30 minutes. Incubation in 0.35 M salt should strip most non-histone DNA-binding proteins from the DNA. The nuclear suspension was then cleared of particulate matter and chromosomal DNA by high-speed centrifugation (100,000 *g* for 60 minutes) at 4°C. Glycerol was added to the supernatant to a final concentration of 20% and the extract was divided into aliquots and stored at −80°C. Typically, yields were ~7.5 μg protein per dissected ovary. Nuclear extracts were also prepared from the carcass fraction of the dissected females. The yields from these preparations were ~1 μg protein per carcass.

Results

The identification of a nurse cell/oocyte transcriptional control region

Previous studies indicated that the *hsp26* sequence −543 to −373 is essential for *hsp26* transcription in nurse cells and oocytes (Cohen and Meselson, 1985). To test if the 171 bp sequence is sufficient to activate a heterologous promoter in such cells, we fused one or two copies of it to the 5′ end of a *lacZ* reporter gene bearing promoter sequences from the *Drosophila Sgs-3* gene (Fig. 2). We found that two copies of the 171 bp sequence strongly activate the *lacZ* reporter in the nurse cells and, more weakly, in the oocytes of ~stage 6 and older egg chambers (see Fig. 2 for summary and Fig. 3 for representative staining pattern). If the staining reaction is carried out for a long time (e.g. overnight) faint staining can be seen in the nurse cells and oocytes of stage 1-6 egg chambers. In no case, however, do we see staining in germ cells prior to egg chamber formation or in any cells outside of the germ line. In contrast to this vigorous activation, one copy of this sequence does not activate the reporter in any examined tissue, ovarian or otherwise (Figs 2 and 3). We conclude that two copies, but not one copy, of the 171 bp sequence are sufficient to stimulate a heterologous promoter in nurse cells and, apparently (see below), oocytes. The requirement for two copies of the 171 bp sequence is suggestive of strong synergistic interactions between multiple copies of a particular DNA control element.

A priori the oocyte staining seen in transformants which carry the *lacZ* reporter linked to two copies of the 171 bp *hsp26* sequence could reflect actual transcription of the *lacZ* reporter in the oocyte or the diffusion of gene products from the nurse cells into the oocyte. However, diffusion should manifest itself as a delay in the time course of oocyte staining versus that of nurse cell staining. We see no such delay; all of the egg chambers exhibiting nurse cell staining also exhibit oocyte staining. Also, nurse cell-specific β-gal staining has been described for a number of enhancer trap lines (Grossniklaus et al., 1989; Fasano and Kerridge, 1988) indicating that *lacZ* transcripts are not intrinsically diffusible. Thus, we interpret the observed oocyte staining to represent transcription in the oocyte and conclude that two copies of the 171 bp sequence activate transcription in oocytes as well as in nurse cells.

Apart from nurse cells and oocytes, two copies of the 171 bp sequence are not active in any examined adult female or male, larval or embryonic tissue. Transcription is not enhanced in salivary glands, even though the *lacZ* reporter contains promoter elements sufficient for a low level of salivary gland expression (Meyerowitz et al., 1987; and data not shown). These results suggest that the 171 bp sequence has an intrinsic specificity for nurse cells and oocytes. For example, the sequence may bind proteins (transcription factors) that, at least as a set, are active exclusively in nurse cells and oocytes. Consistent with this idea, we find that ovaries are enriched for nuclear proteins capable of binding to specific sequence elements within the 171 bp sequence (see below).

To determine whether the 171 bp sequence, like most eucaryotic enhancers (for reviews, see Atchinson, 1988; Serfling et al., 1985), can stimulate transcription when

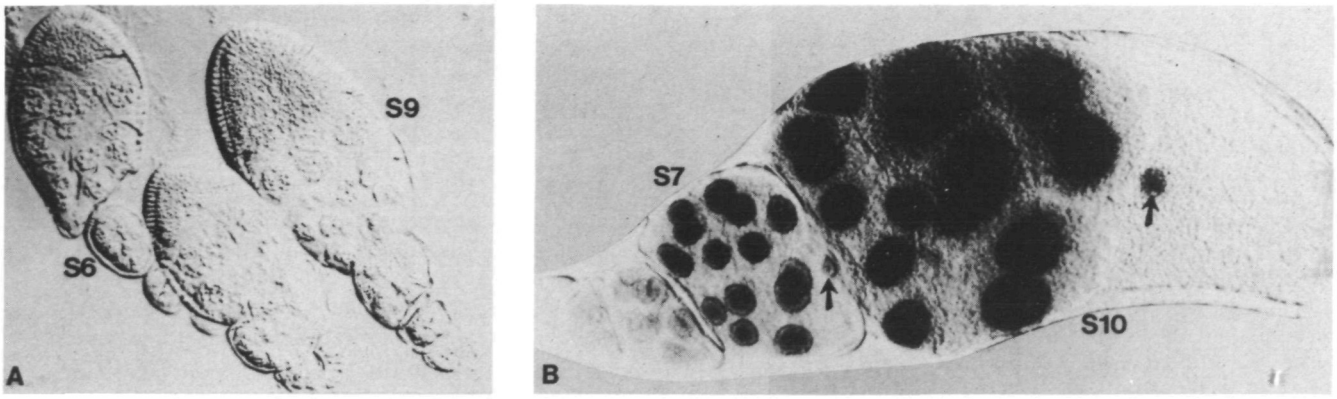


Fig. 3. β-gal staining pattern of transformants carrying *hsp26-lacZ* fusion genes. Ovaries from representative *171-Z* (A) and *2x171-Z* (B) transformants, which carry the *lacZ* reporter gene linked to one and two copies of the 171 bp *hsp26* 5′ sequence, respectively, following histochemical staining for β-gal enzyme activity. No β-gal staining is seen in *171-Z* transformants. In *2x171-Z* transformants, staining is seen in the nurse cells and oocytes of ~stage 6 and older egg chambers. Notice that β-gal staining predominates over nuclei; presumably, the *Sgs-3* portion of the *lacZ* fusion protein directs the protein into the nucleus. Also notice that nurse cell nuclei (non-labeled nuclei) stain more intensely than oocyte nuclei (arrows), especially in older egg chambers. S, egg chamber stage (from King, 1970).

placed downstream of a promoter, we inserted multiple copies of it at a site 6.5 kb downstream of the *lacZ* reporter gene. Four downstream copies of the 171 bp sequence do not stimulate transcription in nurse cells, oocytes or elsewhere (Fig. 2, and data not shown). However, three downstream copies plus one upstream copy of the same sequence activate nurse cell/oocyte transcription (Fig. 2, and data not shown). Recall that one copy of the 171 bp sequence does not activate the reporter gene in nurse cells or oocytes. We therefore conclude that multiple copies of the 171 bp sequence can stimulate transcription when located far downstream of the promoter, provided that one copy of the sequence is located close to the promoter. Because of this provision, we refer to the 171 bp region as a regulatory sequence instead of an enhancer. One possible explanation of this requirement is that the 171 bp sequence contains two control elements, one of which must be close to the promoter and one of which can function close to or far from the promoter.

The nurse cell/oocyte regulatory sequence contains two functionally distinct DNA control elements

To localize the component control element(s) of the 171 bp sequence, we divided it into two overlapping fragments, a 100 bp 5′ fragment (*hsp26* nucleotides −543 to −444) and an 85 bp 3′ fragment (*hsp26* nucleotides −457 to −373), and tested their abilities to stimulate transcription when fused in multiple copies to the *lacZ* reporter. No germ line cells stained in any of the lines which carry these constructs (Fig. 2 and data not shown). These results indicate that the 5′ and 3′ regions each contain a different essential element. Alternatively, there may be only one element, but it may span the breakpoints (i.e., it may extend 5′ to −457 and 3′ to −444). To rule out this possibility, we linked one intact copy of the 171 bp sequence plus one copy of the 3′ 85 bp fragment to the *lacZ* reporter. In each of three lines established, this gene gives a β-gal staining pattern indistinguishable from that of the *lacZ* reporter linked to two copies of the intact 171 bp sequence (Fig. 2, and data not shown). This result shows that the 3′ terminal portion of the 171 bp sequence contains an intact element. Since the 3′ terminal portion alone cannot activate nurse cell/oocyte transcription, it must lack an element present in the 171 bp fragment. We conclude that the 171 bp region contains at least two functionally distinct control elements, one of which lies fully within its 3′ terminal 85 bp and one other which lies within its 5′ terminal 100 bp.

Although 2 or 3 copies of the 5′ 100 bp fragment do not activate the reporter gene in germ cells, they do activate it in a subset of the somatic cells that surround the nurse cell-oocyte cluster, known as border cells (data not shown). Border cell staining is first detected at ~stage 6, when these cells lie at the anterior pole of the egg chamber (Margaritis et al., 1980). Staining persists as these cells migrate posteriorly towards the oocyte nucleus, eventually coming to rest at the nurse cell-oocyte border. No other examined somatic tissue, ovarian or otherwise, stains for β-gal activity. The fact that border cell expression correlates precisely in time to *hsp26* germ line expression suggests that the same temporally specific DNA control element or elements mediate both responses. We cannot rule out the possibility, however, that a temporally specific border cell DNA control element was fortuitously created during the cloning of these constructs.

The nurse cell/oocyte regulatory region contains binding sites for two different ovarian nuclear proteins, ONF-AATAA and ONF-CAACAA

Based on the experiments described thus far, we expected that the *hsp26* 171 bp sequence would contain binding sites for two or more different ovarian transcription factors. To identify such sites and the

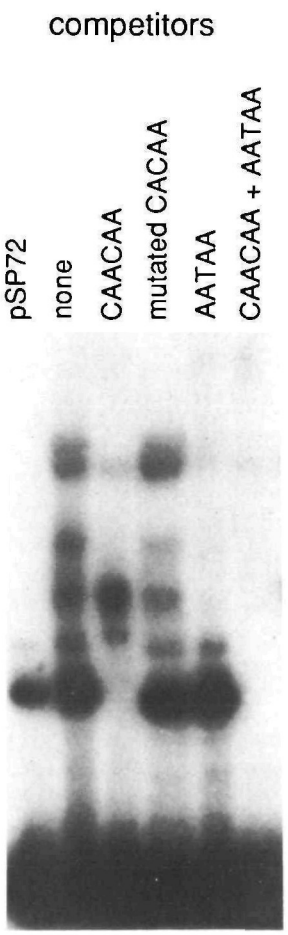


Fig. 4. Gel-shift analysis of protein binding to the *hsp26* nurse cell/oocyte regulatory region. About 1 ng of the *hsp26* nurse cell/oocyte regulatory region (one copy of the 171 bp sequence) was end-labeled and incubated with ~5 μg of the ovarian nuclear extract in the presence of 2 μg of poly d(IC) non-specific competitor DNA and 500 ng of pSP72 plasmid DNA (Promega Biotech.) or 50 ng of various other specific competitor DNAs (indicated at the top of each lane). The band at the bottom of each lane corresponds to free (non-protein-bound) DNA. All other bands correspond to DNA-protein complexes. The copper nuclease footprints of two such bands are shown in Fig. 5. DNA sequences (coding strands): CAACAA, (ctagGACAACAAC-TACaac)$_n$, where upper case letters correspond to footprinted sequence (see Fig. 5), 5′ lower case letters correspond to linker DNA, and 3′ lower case letters correspond to nucleotides that flank the CAACAA element in the endogenous *hsp26* gene, but which are not footprinted; mutated CAACAA, (ctagGAGAAGAAGTACaac)$_n$, where underlined nucleotides indicate mutated residues, other lettering is as indicated for the wild-type CAACAA element; AATAA, caGAATAAAAAAAACTgaatt, where upper case letters correspond to footprinted sequence (see Fig. 5), and lower case letters correspond to linker DNA. Note that pSP72 DNA and the AATAA sequence each compete with the 171 bp sequence for binding to the same factor. Although we have not mapped binding sites within pSP72 DNA, sequence analysis reveals several A-rich regions that resemble the AATAA sequence (data not shown).

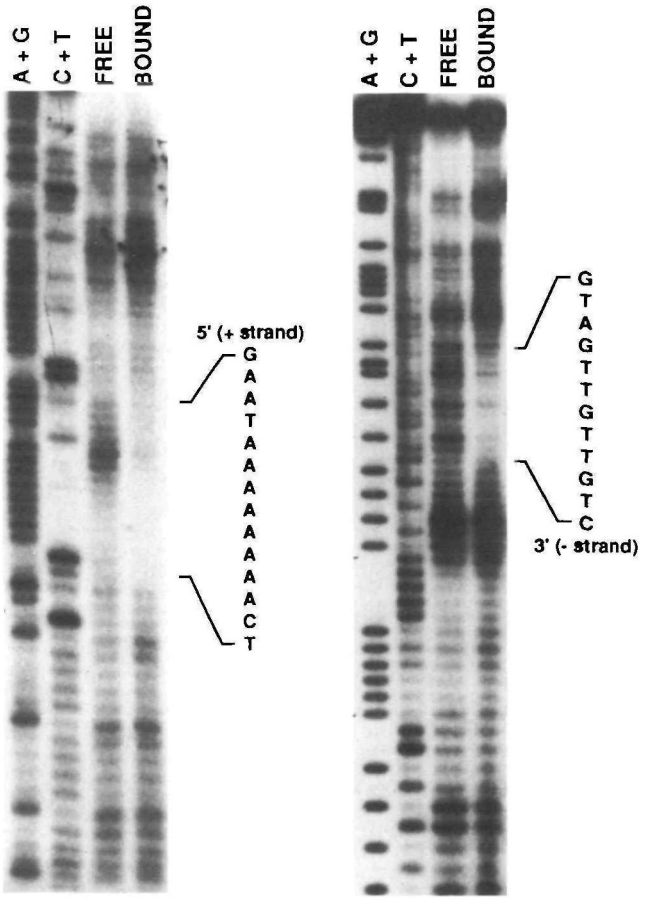


Fig. 5. Copper nuclease footprint analysis of protein binding sites within the *hsp26* nurse cell/oocyte regulatory region. A DNA fragment corresponding to the 171 bp *hsp26* sequence from −543 to −373 was end-labeled on either the coding or non-coding strand and incubated with 5 μg of ovarian nuclear extract as described in Materials and methods. Following gel electrophoresis and treatment of the gel with copper nuclease (see Material and methods), bands corresponding to protein-DNA complexes were individually eluted, denatured, and re-electrophoresed through the denaturing gels shown in the figure. Two different footprints are observed (BOUND lanes), corresponding to *hsp26* nucleotides −436 to −423 (left panel) and −512 to −501 (right panel). The proteins responsible for these footprints are referred to as ONF-AATAA and ONF-CAACAA, respectively. Note that ONF-AATAA equally protects the top (+) and bottom (−) (not shown) strands of its target site, while ONF-CAACAA shows a preference for the bottom (−) strand. Other lanes: (A + G) and (C + T), partial chemical cleavage of the 171 bp binding substrate at purine and pyrimidine residues, respectively; (FREE), DNA not complexed with protein.

corresponding proteins, we used an orthophenanthroline copper nuclease footprinting assay (Kuwabara and Sigman, 1987). This assay differs from standard DNAase I footprinting assays in that in the latter nuclease is added directly to the binding reaction, while in the former nuclease is added after DNA-protein complexes are separated from free DNA by electrophoresis through a low-ionic strength gel. As seen in Fig. 4, the low-ionic strength gel resolves two major and several minor protein-DNA complexes. Copper nuclease treatment of the two major complexes shows that one (Fig. 5, left panel) footprints the sequence (coding strand) GAATAAAAAAAACT (abbreviated AATAA), while the other (Fig. 5, right panel) footprints the sequence (coding strand) GACAA-CAACTAC (abbreviated CAACAA). Each element is present in a single copy in the 171 bp sequence; the AATAA element maps to *hsp26* nucleotides −436 to −423 and the CAACAA element maps to *hsp26* nucleotides −512 to −501. The ovarian nuclear factors

(ONFs) responsible for these footprints are called ONF-AATAA and ONF-CAACAA, respectively.

ONF-CAACAA and ONF-AATAA appear to be the only ovarian nuclear factors that bind to the 171 bp sequence, since none of the major or minor DNA-protein complexes are observed on low ionic strength gels when a mixture of synthetic CAACAA and AATAA elements are added to the binding reaction as non-labeled competitor DNAs (Fig. 4, CAACAA + AATAA lane). The addition of similar amounts of synthetic mutated versions of the CAACAA and/or AATAA elements to the binding reaction does not inhibit ONF-CAACAA or ONF-AATAA binding (Fig. 4, mutated CAACAA lane, and data not shown), indicating that the synthetic CAACAA and AATAA elements inhibit ONF-CAACAA and ONF-AATAA DNA binding in a sequence-specific manner. Moreover, footprint analyses of one of the minor protein-DNA complexes reveals binding to the CAACAA element (data not shown). The fact that more than one of the protein-DNA complexes resolved on the low-ionic strength gels disappears when synthetic CAACAA or AATAA elements are added to the DNA-binding reaction raises the possibility that multiple isoforms of ONF-CAACAA and ONF-AATAA exist in the ovary. Alternatively, ONF-CAACAA and ONF-AATAA may have been partially degraded during the preparation of the nuclear extract.

The ONF-AATAA footprint matches one of two published consensus target sequences for the *Drosophila hunchback (hb)* protein (Stanojevic et al., 1989; Treisman and Desplan, 1989). Since embryos derived from homozygous mutant *hb* mothers develop normally (Lehman and Nusslein-Volhard, 1985), it is unlikely that *hb* and ONF-AATAA are the same protein. In addition, we have been unable to detect *hb* protein on Southwestern blots of our ovarian nuclear extracts using anti-*hb* antibodies (data not shown).

To test directly the roles of the CAACAA and AATAA elements in the activation of nurse cell/oocyte transcription, each site was individually mutated within the context of an otherwise wild-type (two, three or four copies of the 171 bp sequence) 171 bp sequence. Low-ionic strength gel analyses of DNA binding, similar to those described in Fig. 4, show that the CAACAA and AATAA element mutations prevent *in vitro* ONF-CAACAA and ONF-AATAA binding, respectively (data not shown). As summarized in Fig. 2, 171 bp variants containing the CAACAA or AATAA element mutations are incapable of stimulating transcription from the *lacZ* reporter gene. We conclude that the CAACAA and AATAA elements are each essential for the nurse cell/oocyte transcriptional activity of the 171 bp sequence.

ONF-CAACAA and ONF-AATAA binding proteins are highly enriched in ovaries

As a first step in determining the tissue-distribution of ONF-AATAA and ONF-CAACAA, we prepared nuclear extracts from ovarectomized adult females and examined them for CAACAA and AATAA DNA-binding activity. As seen in Fig. 6, lane 5, such extracts contain no detectable AATAA element binding activity. A binding activity similar to ONF-CAACAA is found in such extracts (lanes 5 and 6). However, in contrast to ONF-CAACAA, this non-ovarian factor is heat-labile for DNA-binding (compare lanes 4 and 8). These findings suggest that ONF-AATAA and ONF-CAACAA are ovarian-specific transcription factors. We cannot rule out the possibility, however, that ONF-AATAA and/or ONF-CAACAA are modified in non-ovarian tissues such that their DNA-binding properties, including heat-sensitivity, are altered.

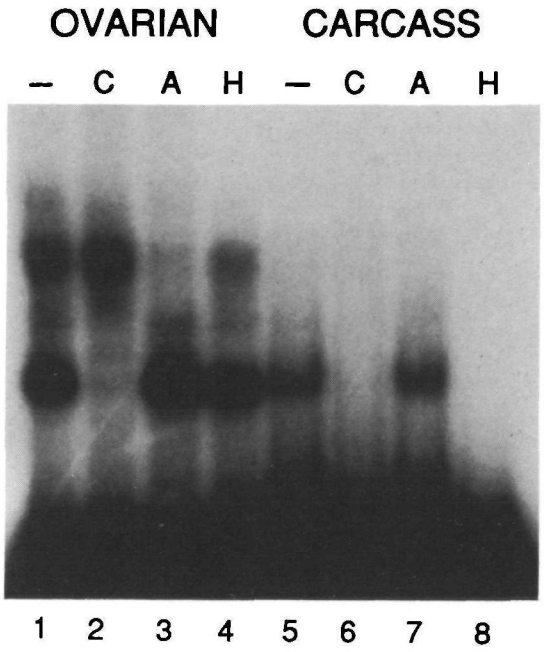


Fig. 6. ONF-AATAA and ONF-CAACAA are enriched in ovaries. The 171 bp *hsp26* sequence was end-labeled and incubated with ~5 μg nuclear extract prepared from the ovarian (lanes 1-4) or carcass fractions (lanes 5-8) of dissected adult females. In the lanes labeled H, the extract was heated at 90°C for 15 minutes prior to adding the DNA. Note that the heat treatment specifically destroys the carcass CAACAA element binding activity. All lanes contained 2 μg of poly-d(IC) as a non-labeled competitor DNA. Other competitor DNAs were as follows: C lanes, 50 ng of the synthetic CAACAA element; A lanes, 50 ng of the synthetic AATAA element. The sequences of the synthetic CAACAA and AATAA elements and other details of the binding reactions are given in Fig. 4, and in Materials and methods.

Nurse cell/oocyte transcriptional activity is dependent on multiple ONF-CAACAA and ONF-AATAA binding sites

To determine how many copies of the ONF-AATAA and ONF-CAACAA binding site are required for nurse cell/oocyte transcriptional activity, we linked an intact copy of the 171 bp sequence and two copies of the 171 bp sequence mutated at either AATAA or CAACAA to the *lacZ* reporter to create *171/2xmutAATAA-Z* and *171/2xmutCAACAA-Z*, respectively (Fig. 2). 11 of 12

lines carrying the *171/2xmutAATAA-Z* construct showed no nurse cell or oocyte staining; in the twelfth line, stage 10 nurse cells and oocytes stained weakly (summarized in Fig. 2). In contrast, 10 of 11 lines carrying the *171/2xmutCAACAA-Z* construct showed nurse cell and oocyte staining, although the level of staining was reduced compared to that seen with transformants that carry the *lacZ* reporter linked to two intact copies of the 171 bp sequence (summarized in Fig. 2). We conclude from these data that nurse cell/oocyte transcriptional activity requires only one functional CAACAA element but more than one AATAA element. However, two copies of each element are required for maximal activity.

Because the *hsp26* gene contains only one copy of the 171 bp sequence, we wondered if additional ONF-AATAA and ONF-CAACAA binding sites are found outside of this region, within the general proximity of the *hsp26* promoter. We find a 12 out of 14 match to the ONF-AATAA binding site at position −615, and a perfect match to the ONF-CAACAA binding site at position −295 (the 171 bp sequence corresponds to the sequence −543 to −373). No other striking homologies were found between the 171 bp sequence and the −741 to +14 region of the *hsp26* gene, which previous studies identified as the minimal sequence required for nurse cell/oocyte transcription (Cohen and Meselson, 1985). The −615 sequence appears to be a bona fide ONF-AATAA binding site, since a 203 bp fragment that covers this sequence, but which does not overlap with the 171 bp fragment, specifically competes with the latter for ONF-AATAA binding (data not shown). Moreover, one copy of the 203 bp sequence plus one copy of the 171 bp sequence activates the *lacZ* reporter in nurse cells and oocytes, albeit at a level ~3-fold lower than that seen with two copies of the 171 bp sequence (Fig. 2). The −295 CAACAA element appears to be a bona fide ONF-CAACAA binding site, since the crude ovarian nuclear extract footprints precisely this sequence (data not shown). It seems likely, therefore, that multiple copies of the ONF-AATAA and ONF-CAACAA binding sites are required for the transcription of the endogenous *hsp26* gene in nurse cells and oocytes as well as for the transcription of the *lacZ* reporter in such cells.

Discussion

Two copies of a 171 bp sequence from the hsp26 *gene function as a nurse cell/oocyte-specific transcriptional regulator*

Our studies show that two copies of a 171 bp sequence from the 5′ flanking region of the *hsp26* gene function as a stage-specific female germ line transcriptional regulator when linked to a *lacZ* reporter gene. Apart from the germ line, the transcriptional regulator is not active in any examined adult female, male, or embryonic cell type or tissue, including pole cells and testes. The strong specificity of this regulator for the female germ line should make it a good tool for over- or ectopically-expressing cloned genes in the egg/early embryo, especially when the expression of the cloned gene in other tissues may be lethal.

Although the *hsp26* nurse cell/oocyte transcriptional regulator, when linked to the *lacZ* reporter, leads to a greater accumulation of β-gal staining activity in nurse cells than in oocytes, it is conceivable that its regulatory proteins are equally abundant (and active) in both cell types. This follows from the fact that nurse cells are highly polyploid, while oocytes are diploid. Indeed, based solely on chromosome ploidy differences (King, 1970; Mahowald and Kambysellis, 1980), a stage 6 nurse cell should produce ~125 times more β-gal activity than a stage 6 oocyte, while a stage 10 nurse cell would produce ~500 times more β-gal activity than a stage 10 oocyte. That stage 6 nurse cells produce only ~3-fold more β-gal activity than a stage 6 oocyte suggests that one or more required transcription factors are limiting in stage 6 germ cells. Such factors appear to increase in abundance as oogenesis proceeds. By stage 8, for example, nurse cells stain 50- to 100-fold more intensely than do oocytes. This increase may be due to the synthesis of the factors in nurse cells and oocytes or to the activation of pre-existing pools of factor by extrinsic cues, e.g., interactions with the somatic follicle cells that surround the nurse cell-oocyte cluster.

Regulator substructure

Site-directed mutagenesis of the *hsp26* nurse cell/oocyte transcriptional regulator shows that its activity is dependent on intact copies of the CAACAA and AATAA elements. At least one of these elements must be present in multiple copies: one copy of the 171 bp fragment possesses no transcriptional activity, while two copies of the same sequence does. Additional copies of the CAACAA and AATAA elements are found in the upstream region of the *hsp26* gene, outside of the 171 bp sequence, consistent with the idea that the nurse cell/oocyte transcription of the endogenous *hsp26* gene, like the transcription of the *lacZ* reporter, is dependent on multiple copies each of the CAACAA and AATAA elements. Multiple copies of these elements are also found in the promoter regions of some other genes expressed in nurse cells and oocytes including *bicoid* and *hunchback* (unpublished observations), suggesting that they are responsible for the nurse cell/oocyte transcription of many different genes.

The requirement for multiple copies of a particular DNA control element is well-documented both in procaryotic and eucaryotic systems (McKnight and Tjian, 1986; Maniatis et al., 1987; Jones et al., 1988). In the case of the λ repressor gene, the synergy observed between the O_R1 and O_R2 operator sites is explicable by the finding that repressor protein binds to these sites cooperatively (Meyer et al., 1980; Meyer and Ptashne, 1980). Cooperative binding has also been reported for several eucaryotic transcription factors (Lebowitz et al., 1989; Davidson et al., 1988; Tsai et al., 1989; Topol et al., 1985). However, preliminary studies indicate that neither ONF-AATAA nor ONF-CAACAA bind to their sites cooperatively: nonligated copies of synthetic

CAACAA and AATAA elements compete with the 171 bp sequence for ONF-AATAA and ONF-CAAACAA binding, respectively, as efficiently as ligated copies of the same elements (L. H. Frank, unpublished). It is conceivable that two ONF-AATAA molecules (or two ONF-CAACAA molecules) bind to two DNA sites independently of each other, but cooperate in some other step of transcriptional activation. Two molecules could, for example, simultaneously touch and stabilize the binding of a third molecule, such as TFIID. This sort of cooperativity, termed promiscuous cooperativity (Ptashne, 1988; Carey et al., 1990a; Lin et al., 1990), and cooperative binding are not mutually exclusive.

In addition to the strong homosynergistic interactions that we see between multiple CAACAA elements and/or between multiple AATAA elements, we see strong heterosynergistic interactions between CAACAA and AATAA elements. A DNA fragment containing two CAACAA elements plus two AATAA elements possesses strong transcriptional activity, while as many as four copies of the same fragment specifically mutated at either the CAACAA or AATAA element have no detectable transcriptional activity. One interpretation of this finding is that ONF-AATAA and ONF-CAACAA are functionally distinct transcription factors, i.e., each factor may carry out a different essential step in the activation of transcription. For example, one factor may contact TFIID, while the other factor may contact TFIIB or some other component of the basal transcriptional machinery. Although some transcription factors, such as the yeast GAL4 protein (Carey et al., 1990b; also see Mitchell and Tjian, 1989), can on their own activate the basal transcription machinery, it may be that such factors interact with multiple components of this machinery (Kelleher et al., 1990; Berger et al., 1990; Lewin, 1990). Alternatively, ONF-AATAA and ONF-CAACAA may be functionally equivalent factors (i.e., each may make the same contacts with the basal transcriptional machinery), but each factor may be present in limiting quantities in the ovary such that no one factor can make all of the necessary contacts.

Based on our DNA-binding studies, ONF-AATAA and ONF-CAACAA appear to be restricted to the ovaries of adult females. A CAACAA element binding factor was detected in non-ovarian cells, but in contrast to ONF-CAACAA, this factor was heat-labile. Whether ONF-AATAA and/or ONF-CAACAA is restricted to the germ component of the ovaries (i.e., nurse cells and oocytes) is an open question. Based on the characterization of other tissue-specific regulatory regions and enhancers (for examples, see Scheidereit et al., 1988; Bodner et al., 1988; Frain et al., 1989), we expect that at least one of the two factors will be enriched in such cells. Consistent with this expectation, we have recently cloned a gene that possesses CAACAA-element binding activity *in vitro* and have shown that DNA from the 5′ flanking region of this gene activates a *lacZ* reporter gene in nurse cells and oocytes much more vigorously than it does in any other ovarian or non-ovarian cell type of adult females (L. H. Frank, unpublished). A full appreciation of the underlying regulatory logic of germ cell-specific transcription, however, will require a more detailed analysis of ONF-CAACAA as well as the cloning and characterization of ONF-AATAA.

We thank Dale Dorsett for advice on the preparation of nuclear extracts, Paul Macdonald and Mike Levine for the anti-hunchback antibody, K. Calame and E. Kakkis for help with the copper nuclease footprinting procedure, and Vicki Corbin, Tom Serano, and Richard Mann for comments on the manuscript. This work was supported by grants from the March of Dimes and the NIH to R.S.C.

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(Accepted 29 October 1991)