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THE VITELLOGENIN GENE FAMILY OF *AEDES AEGYPTI*

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We have been interested in identifying genes that play a role in reproduction of the mosquito Aedes aegypti. *Our interests are currently focused on the vitellogenin genes which in the mosquito are expressed only in the fat body in response to the insect steroid hormone, 20-hydroxyecdysone. Four of the five vitellogenin genes in the genome have been cloned. We have examined the relationships between these genes and find that they form a small gene family exhibiting different levels of relationship.*

Egg yolk contains all of the nutrients needed for the development of the embryo. The major source of amino acids in egg yolk comes from proteins known as vitellogenins, which can comprise up to 90% of the protein of the yolk. The native vitellogenins are large proteins, with Mr about 250 to 500 kD, that are synthesized in extra-oocytic tissues (Hagedorn & Kunkel, 1979). In vertebrates vitellogenins are made in the liver under the control of estrogen, while in insects the vitellogenins are made in the fat body under the control of either juvenile hormone or 20-hydroxyecdysone as discussed below.

The hormonal control of vitellogenin synthesis in insects varies. In cockroaches (Engelmann, 1979) and locusts (Chen et al., 1979; Wyatt et al., 1983) juvenile hormone controls vitellogenin synthesis, while in many cyclorraphad flies, and the mosquito, both juvenile hormone and 20-hydroxyecdysone appear to be involved in controlling vitellogenin synthesis (Hagedorn et al., 1975; Postlethwait & Handler, 1979; Bownes, 1982; Hagedorn, 1985). The situation in Drosophila is complicated by the fact that both the fat body and the follicle cells surrounding the oocyte make vitellogenin (Brennan et al., 1982; Isaac & Bownes, 1982). It has been suggested that in Drosophila 20-hydroxyecdysone controls vitellogenin synthesis by the fat body, while juvenile hormone controls vitellogenin synthesis by the ovarian follicle cells (Jowett & Postlethwait, 1980). However, recent work has shown that juvenile hormone treatment can stimulate the production of 20-hydroxyecdysone (Schwartz et al., 1985). Postlethwait & Kunert (1985) conclude from their study of the endocrine control of the vitellogenin gene that juvenile hormone also plays a role in the regulation of vitellogenin synthesis by the fat body by regulating the levels of the 20-hydroxyecdysone receptor. We believe that juvenile hormone may have a similar role in the mosquito (Martinez & Hagedorn, 1987).

Because large amounts of vitellogenin are made in response to hormonal signals, the vitellogenin genes have been found to be favorable material for studying how hormones affect gene expression. Among the lower vertebrates study of the vitellogenin genes is most advanced in the toad *Xenopus laevis* (Martin et al., 1986; Whali et al., 1981). Among insects, the most extensive investigation of vitellogenin gene structure at the molecular level has been achieved with *Drosophila melanogaster.*

In the toad, a family of four vitellogenin genes has been identified. The mRNA transcribed from the two type A genes differs from that encoded by the two type B genes in 20 percent of its nucleotide sequence. The coding sequences of these genes are interrupted by many (33) introns that show considerable homology in position, but not in sequence. Within each class of vitellogenin genes, the exons have diverged only by about five percent, implying an evolutionarily ancient duplication of the ancestral vitellogenin gene to give class A and class B genes which were more recently duplicated (Whali et al., 1981).

In *Drosophila* there are three vitellogenin subunits called YP-1, 2 and 3 that have apparent molecular weights of 46, 45, and 44 kD. The vitellogenin genes were cloned by Barnett et al., (1980) and Riddell et al., (1981).

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All three vitellogenin genes are on the X chromosome. The YP-1 and 2 genes are found in opposite orientation within 1.2 kb of one another, while YP-3 is over 1000 kb away from them. There is only one copy of each gene on the chromosome and these genes are not amplified during development (Barnett & Wensink, 1981). A single short intron is present in YP-1 and 2, while YP-3 has two short introns. The nucleotide sequences of YP-1 and 2 have been determined (Hung & Wensink, 1981; 1983) and they show 63% identity. The available evidence suggests that YP 1 and 2 arose by gene duplication followed by limited divergence. Two independent cis-acting control elements were found to be essential for the expression of YP-1 and 2 in the fat body and follicle cells (Garabedian et al., 1985). A small 5' segment of DNA, 125 bp long, was found to enhance fat body expression of YP-1 (Garabedian et al., 1986).

Two genes have been cloned in the locust, *Locusta migratoria* (Wyatt et al., 1984). These two genes show little cross hybridization and few common restriction sites. One copy of each gene is present in the genome. They are coordinately expressed during vitellogenesis (Dhadialla et al., 1987).

Based on studies of the immunochemical relationships between the vitellogenins of various animals, the general conclusion has been that vitellogenins as a group are rapidly evolving molecules designed mainly as sources of amino acids for the developing embryo (Hagedorn & Kunkel, 1979). Thus, immunological cross-reactivity is quickly lost outside of the family level of relationship. This conclusion is also obtained when vitellogenin genes of a single species are compared. For example in *Drosophila*, although YP-1 and 2 presumably represent recently duplicated genes, their nucleotide sequences differ by 37%. Similar conclusions have been reached after studying the vitellogenin genes of the locust and the toad. Despite this, the vitellogenins share some basic characteristics, such as size, presence of carbohydrate and lipid, and ability to enter the oocyte by micropinocytosis. Hybridization experiments suggesting that vitellogenins from widely separated taxa have unexpected homology are of questionable validity (James et al., 1982). Clearly there is much to be learned about these molecules that might be useful in establishing relationships among animals (Kunkel et al., 1976) and in understanding the evolution of rapidly evolving molecules.

MATERIALS AND METHODS

Mosquitoes were derived from the Rock strain of *Aedes aegypti*. They were reared under standard conditions (Shapiro & Hagedorn, 1982).

Recombinant DNA methods were as described by Gemmill et al. (1986). High stringency Southern hybridizations were done in 1.8X SSC at 65 degrees, using probes prepared by primer extension labelling of cloned DNA fragments isolated from low melting temperature agarose gels (Feinberg & Vogelstein, 1984).

RESULTS

Two candidate vitellogenin genes of *A. aegypti* were isolated from a Charon 4 genomic library by selecting clones that hybridized to an abundant 6.5 kb RNA that was present in vitellogenic females but absent in males or non-blood-fed females (Gemmill et al., 1986). These two clones, A1 (previously 403-1) and C (previously 421-2), crosshybridized over a region spanning approximately 6.5 kb. Detailed restriction maps, however, showed that they represent distinct genes. The approximate extent of the coding region was determined by hybridization of subcloned restriction fragments to vitellogenin mRNA on Northern blots. Part of the coding region of A1 was inserted into an expression vector, and the resulting polypeptide reacted specifically with antibodies to vitellogenin, thus demonstrating that the cloned gene actually represented a vitellogenin gene. Given the size of the coding region (6500 nucleotides), and the size of the large vitellogenin subunit (190 kD), it seems unlikely that there are large introns in these genes, assuming that we have cloned the entire gene sequence.

We have recently identified two additional vitellogenin genes, giving us a total of four cloned genes out of the five which we believe to be present in the *A. aegypti* genome (Gemmill et al., 1986). An *A. aegypti* library in EMBL-4 was constructed and screened for sequences homologous to the vitellogenin gene using a subcloned 1.5 kb Eco RI fragment of gene A1 (stippled in Fig. 1) labelled by nick translation. About 50,000 plaques were screened, or approximately 2/3 of a genome. Four recombinant phages that hybridized to this fragment were isolated and mapped, resulting in the identification of two new genes. As shown in Fig. 1, clone A2 had most of the same restriction sites as gene A1 but lacked the 3' end of the gene.

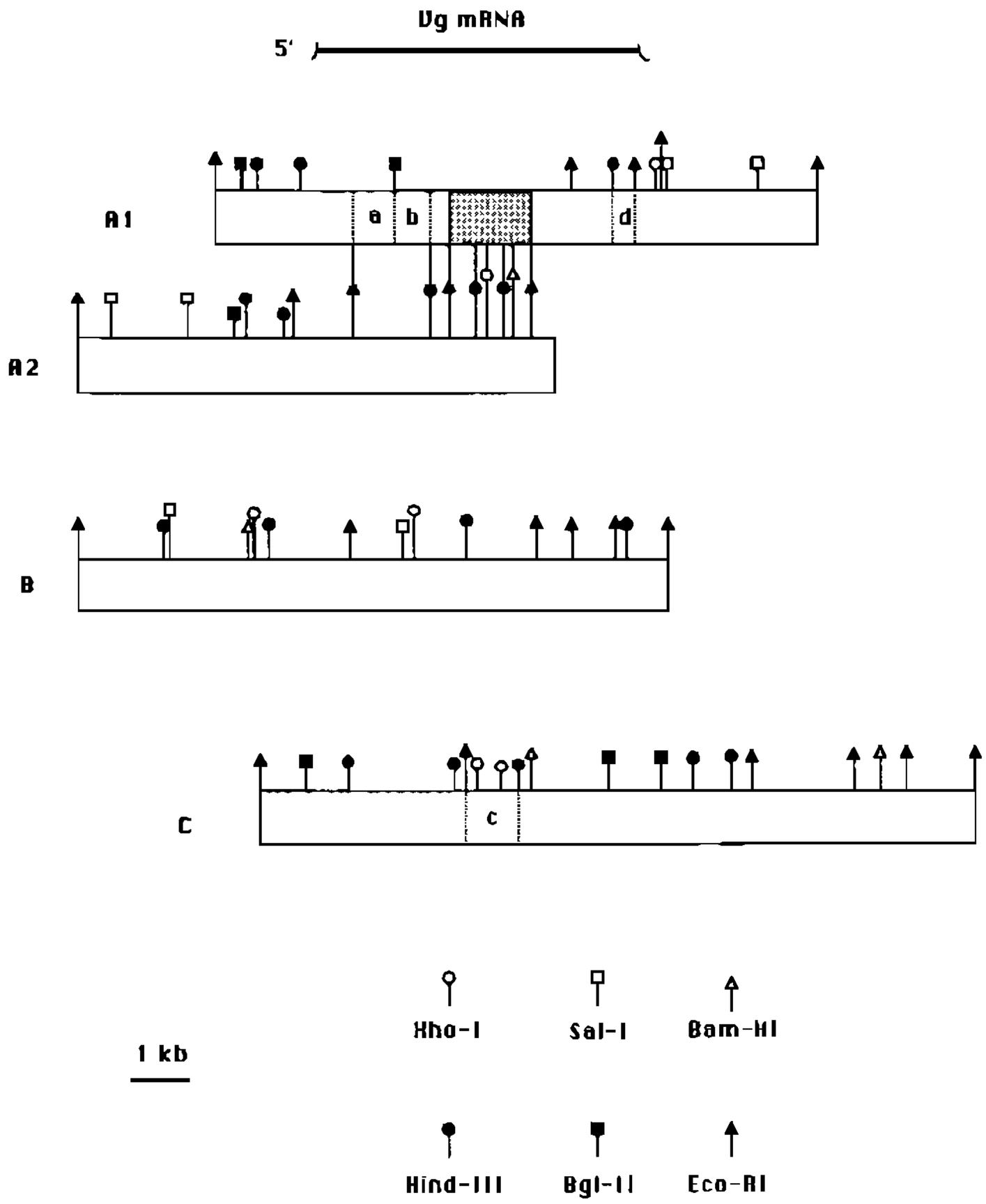

Fig. 1: Restriction maps of *A. aegypti* vitellogenin gene clones.

Clone B had no restriction sites in common with either A1 or C.

In order to determine the degree of conservation of the vitellogenin genes, small (200-800 bp) fragments of two of the genes, A1 and C, were used as probes in high stringency Southern hybridizations against all four cloned vitellogenin genes. Equal amounts of the four phage DNAs were digested with restriction enzymes, separated on an agarose gel, and transferred to a nylon membrane which could be probed repeatedly. Fragments spanning the entire length of the coding regions of A1 and C were used as probes. We found that the two genes A1 and A2 are very highly homologous, and that B is slightly less homologous to them. However, C shows only weak homology to the other genes at high stringency. For example, when the A1a fragment (see Fig. 1) is used as a probe, the 1.8 kb Eco RI fragments of A1 and A2 hybridize with the same intensity, while the 3.1 kb fragment of B hybridizes almost as strongly (Fig. 2a). When fragment A1b is used as a probe, A1 and A2 again show no difference, but B is less homologous in that region (Fig. 2b). Neither one of these probes shows significant hybridization with clone C. Conversely, when fragment c of gene C is used as a probe, in hybridizes only back to clone C and not to the other three genes (Fig. 2c). The most highly conserved region among all four genes appears to be near their 3' ends. In Fig. 2d, fragment d of gene A1 hybridizes to A1, B, and C. Clone A2 does not contain the 3' end of the gene and so does not show any hybridization.

Because gene C shows only weak homology to gene A1, even in the region of conserved restriction sites reported by Gemmill et al. (1986), we reexamined this region by finer mapping of subclones. This mapping showed that the distances between the restriction sites in this region are different in the two genes. Although gene C shows considerable divergence from gene A1, which is known to be expressed, it hybridized on a Northern transfer to the same 6.5 kb RNA (Fig. 3). We therefore believe that it, too, is an expressed gene.

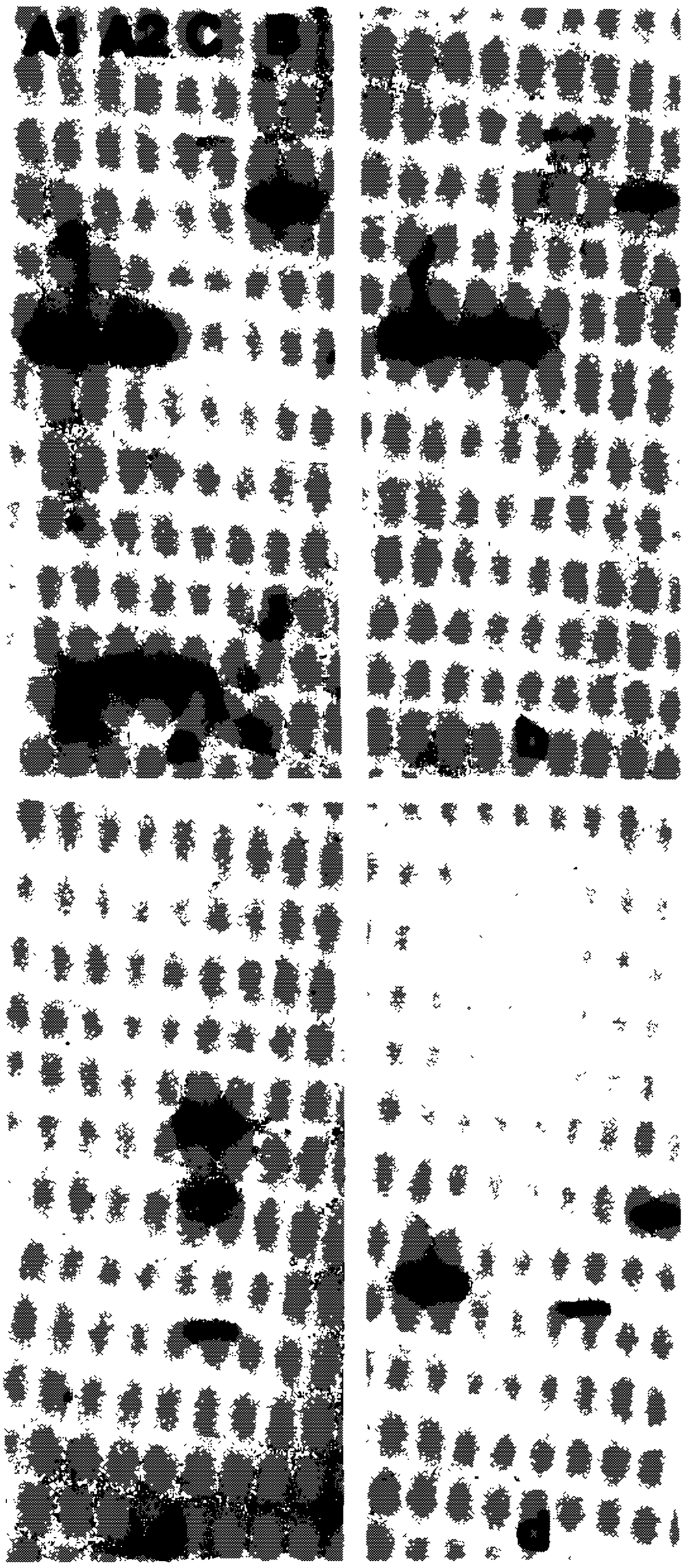

Fig. 2: Homology between four vitellogenin genes. Equal amounts of DNA from clones A1, A2, B and C were digested with Eco RI (A1 and A2) or Bgl II and Hind III (B and C; digestion of clone C was incomplete). Digested DNA was transferred and probed as described in Gemmill et al. (1986). The restriction endonuclease fragments identified with small letters a-d in Fig. 1 correspond to the probes used in experiments a-d of this figure.

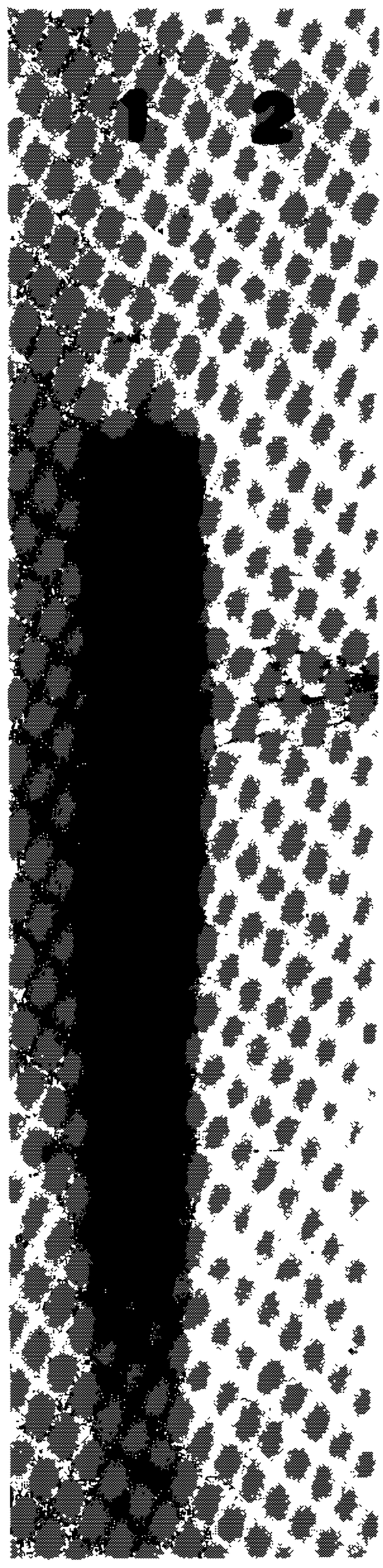

Fig. 3. Hybridization of DNA from clone C to poly(A)$^+$ RNA. Poly(A)$^+$ RNA from 1) blood-fed-females and 2) nonblood-fed females was isolated, fractionated and transferred as described in Gemmill et al. (1986), and hybridized with nick translated DNA from clone C.

DISCUSSION

We therefore have isolated and mapped 4 of the 5 vitellogenin genes that we believe are present. It is interesting to note that two of the four genes have very similar restriction sites, and the other two have no sites in common with the other three. The vitellogenin genes in *A. aegypti* therefore appear to be present in multiple copies, a situation that is similar to that seen in Xenopus in which two different vitellogenin genes (A and B) are present with

several different copies of each in the genome (Whali et al., 1981). In *A. aegypti,* the gene family shows three different levels of divergence, represented by A1 and A2, B, and C.

The presence of gene families could have fuctional consequences. If all the members of a gene family are expressed, and we know that at least two of the *A. aegypti* genes are, then they could have evolved different sensitivities to hormonal stimulation. For example, gene A1 could be expressed at low and high concentrations of 20-hydroxyecdysone, while gene C might only respond to high concentrations. The members of a gene family might also evolve different functional roles. In the case of vitellogenin, the product of one gene might be degraded during early embryonic development, while another might not be degraded until later. There is, in fact, a follicle cell product in *Hyalophora cecropia* called microvitellogenin (Telfer et al., 1980) that appears to be degraded before vitellogenin. In this case it is not known if microvitellogenin is a member of a vitellogenin gene family.

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