causing depolarization of the type B cell. Analysis of protein phosphorylation in the type B cell, particularly in relation to the training procedures described, may eventually suggest specific biochemical steps within the associative learning procedure for *Hermissenda* and possibly for more evolved species.

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7. Photoreceptors had axons cut either at their exit point from the eye (leaving essentially the isolated somata) or ~ 30 to 40 μm from the exit point. These lesions (*8*) eliminated all synaptic interactions and impulses of the photoreceptors. Input resistance usually increased slightly for such cells. Occasionally, residual impulse activity was observed with the cut 40 μm from the exit point (Fig. 1C). Current and voltage records were otherwise identical for these two types of preparations.
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10. Absolute values of holding potential are given assuming an RP of −55 mV; RP was not measured precisely each time but there was a variation of ± 10 mV when it was determined.
11. The maximum frequency response of this instrument (100 Hz for 1 cm, 40 Hz for 4 cm) was adequate for the relatively slow current and voltage responses of interest.
12. A photosensitive peak at 509 nm [D. L. Alkon, in *Neural Principles of Vision*, F. Zettler and R. Weiller, Eds. (Springer-Verlag, Berlin, 1976), p. 410] has been determined for type B cells. Because the light source used here emitted only a small percentage of its light at or near this wavelength, high source intensities were necessary (10^5 to 10^6 erg cm^{-2} sec^{-1}). These intensities (4-second steps) caused no measurable (± 0.1°C) changes of bath temperature; 30-second light steps (10^6 erg cm^{-2} sec^{-1}) caused a photoreceptor LLD response (Fig. 1A) comparable to that elicited (*8*) by a quartz-iodide source (10^4 erg cm^{-2} sec^{-1}) with no measured temperature change. Light filtered by a 500 ± 10 nm narrow-band interference filter (Oriel, Stamford, Conn.) produced, at sufficient intensities (10^1 to 10^3 erg cm^{-2} sec^{-1}), essentially identical photoreceptor voltage and current responses.
13. Artificial seawater (ASW) contained Na^+, 482.5 m*M*; K^+, 10 m*M*; Mg^{2+}, 55 m*M*; Ca^{2+}, 10 m*M*; Cl^-, 620 m*M*; and tris buffer, 10 m*M* (*p*H 7.2).
14. Although light-induced Ca^{2+} inward currents were previously not clearly observable in other photoreceptors with voltage clamp experiments, their presence has been inferred or monitored with other techniques by previous workers [H. M. Brown, S. Hagiwara, H. Koike, R. M. Meech, *J. Physiol. (London)* **208**, 385 (1970); J. E. Brown and J. R. Blinks, *J. Gen. Physiol.* **64**, 643 (1974)]. That the sustained light-induced inward current is carried by Ca^{2+} across the type B photoreceptor membrane was confirmed by the observation that it was present in 120 m*M* K^+, Na^+-free ASW at the K^+ reversed potential (Figs. 1B, bottom, and 2C), as well as in Na^+-free ASW at the approximate reversal potentials for Na^+ and Cl^- currents (Fig. 1, D and E, and Fig. 2, A and B).
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5-Bromodeoxyuridine Inhibits Sequence Changes Within Inverted Repeat DNA During Embryogenesis

Abstract. *Previous studies on the genome of* Strongylocentrotus purpuratus *sea urchin have shown that changes in the nucleotide sequence of inverted repeat sequences occur during embryogenesis. The present study indicates that these sequence changes fail to occur when the embryos are raised in the presence of 5-bromodeoxyuridine. This drug is an analog of thymidine, is incorporated into the DNA during embryogenesis, and inhibits cell differentiation in these embryos.*

Previous experiments on *Strongylocentrotus purpuratus* sea urchin embryos suggest that there are sequence changes during embryogenesis within inverted repeat DNA sequences in the genome (*1*); we now show that these sequence changes do not occur when sea urchin embryos are cultured in the presence of 5-bromodeoxyuridine (BrdU).

Incorporation of the thymidine analog BrdU into DNA arrests differentiation in many biological cells (*2*), including *S. purpuratus* embryos (*3, 4*). With 50 μg of BrdU per milliliter of seawater media present from the time of fertilization of these embryos, development is altered at gastrulation (*3*), numerous anatomical changes occur prior to the completion of blastulation (*3, 4*), 30*S* to 60*S* duplex DNA pieces accumulate during the cleavage period of development (*5–7*), and BrdU replaces as much as 41 percent of the thymidine in the embryo DNA by the time that development has reached morula stage (*7*).

We have tested inverted repeat DNA sequences for sequence changes during embryogenesis by comparing the bulk adjacent inverted repeats taken from *S. purpuratus* sea urchin embryos at different stages of development (*1*). When isolated, sheared, sea urchin DNA is denatured and renatured at $< 10^{-4}$ C_0t (moles of nucleotide per liter times time in seconds) and the duplex DNA is collected on hydroxyapatite, the final DNA preparation recovered after three rounds of this renaturation is largely hairpin-like in structure (*1*); the hairpins are formed by foldback with subsequent intrastrand hydrogen-bonding of those homologous sequences that are inverted relative to each other and are adjacent or nearly so on the same strand of DNA (*1, 8, 9*). Folding of isolated strands of inverted repeat DNA in vitro causes base-pair mismatches to be formed at points on the stems of the hairpins wherever there are departures in the homology of the left and right arms of the individual adjacent

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inverted repeats that are present in vivo (*1*). Measurement of the distribution of mismatch positions on bulk hairpin DNA has allowed us to assay for sequence changes during embryogenesis within left or right arms (or both) of these adjacent inverted repeats (*1*).

The distribution of lengths of hairpin DNA derived, as described above, from *S. purpuratus* embryo DNA was the same for each of the sets of hairpins derived from the bulk DNA's of morula, blastula, and gastrula stage embryos; the single-stranded ends of these hairpins had been trimmed with *Escherichia coli* 3′,5′ exonuclease VII (*10*) prior to the developmental stage-specific comparison of the distribution of lengths of the hairpins on electrophoretic gels (*1*). However, when the sets of hairpins derived from the same three embryological stages were incubated with single-strand-specific S1 endonuclease from *Aspergillus oryzae* under conditions that did not cut the strands of duplex bacteriophage DNA, the average length of the resulting S1 pieces was on the order of ten times shorter than the average length of the exonuclease VII-trimmed hairpins (*1*). This suggested that the majority of these hairpin stems contain S1 nuclease-sensitive base-pair mismatch sites. Our interest has focused on the finding that the average length of the S1 pieces derived from hairpins is developmental stage-specific: the average length of S1 pieces is about 84 base pairs for morula stage, 70 base pairs for blastula stage, and 57 base pairs for gastrula stage (Fig. 1a). In view of the constancy during embryogenesis of the length distribution of the exonuclease VII-trimmed hairpins (*1*), the developmental stage-specific shift in the average length of the S1 pieces derived from hairpin stems suggests (*1*) (Fig. 1a) that during early embryogenesis there are sequence changes in vivo within the majority of those adjacent inverted repeat sequences, which in vitro form hairpins whose stems contain S1 sites. The average distance between S1 sites on the hairpins is reduced as embryogenesis proceeds.

Comparison of the length distributions of S1 pieces resulting from the sets of hairpins derived from bulk DNA's of different stages of embryos raised in seawater containing BrdU is shown in Fig. 1b. The predominant size of the S1 pieces is about 110 base pairs for morula, 105 base pairs for blastula, and 105 base pairs for gastrula hairpins. In these experiments, the gastrula stage embryos had been allowed to develop until they had the appearance of BrdU-altered gastrulae; additional time for development in BrdU did not change the distribution of lengths of the S1 pieces. It is apparent that the large shift in the distribution of lengths of S1 pieces seen in the control case (embryos not treated with BrdU) (Fig. 1a) does not occur in BrdU-treated embryos. These *S. purpuratus* embryos incorporate BrdU into nuclear DNA and synthesize normal amounts of DNA per cell division until at least early gastrula stage (*7, 11*). Although a longer developmental time period is required compared with control embryos, at least early gastrula stage is achieved by the majority of a population of embryos grown in seawater medium containing 50 μg of BrdU per milliliter of medium (*12*).

We previously examined the distribution of lengths of S1 pieces resulting from the cutting at base-pair mismatch sites of the stems of a subpopulation of hairpins that could be distinguished from the general population of hairpins; this subpopulation was derived from inverted repeat sequences that had been methylated in vivo during embryogenesis (*1*). In these experiments, hairpin DNA was isolated from embryo bulk DNA which had been labeled in vivo with [^{3}H]-methionine and ^{32}P. [^{3}H]Methionine la-

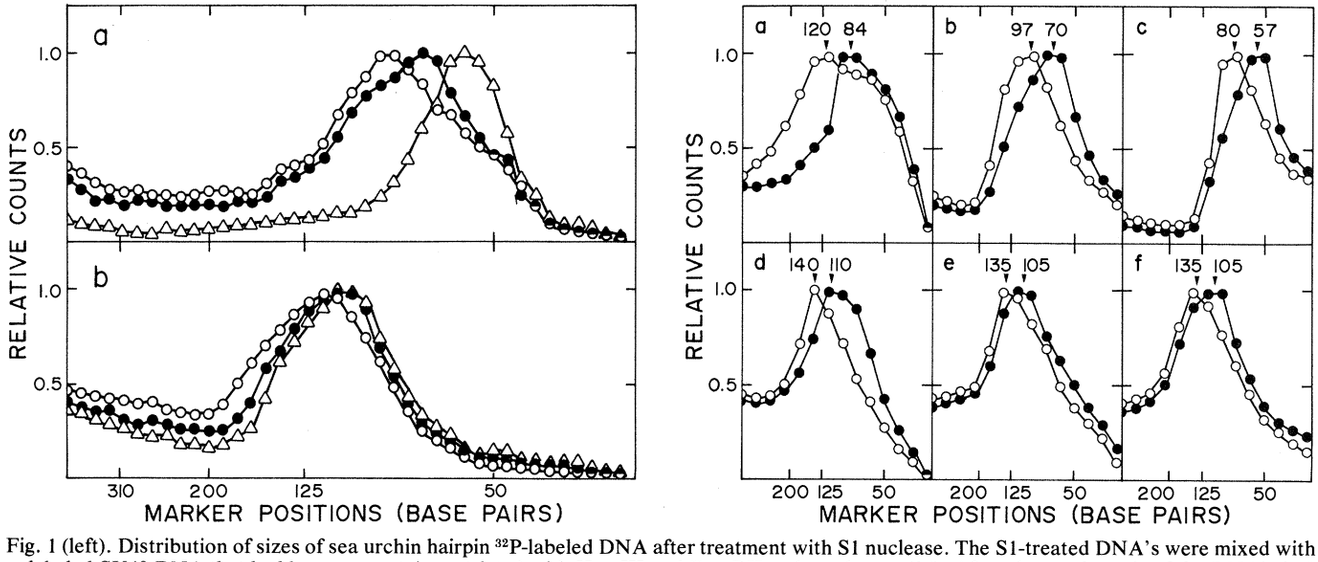

Fig. 1 (left). Distribution of sizes of sea urchin hairpin ^{32}P-labeled DNA after treatment with S1 nuclease. The S1-treated DNA's were mixed with unlabeled SV40 DNA that had been separately restricted with Hae III and Eco RII endonucleases (*14*). After electrophoresis of the DNA's in 7 percent acrylamide, the gels were stained with ethidium bromide to identify, by ultraviolet-induced fluorescence, the SV40 marker positions; the gels were then sliced and counted. The corresponding gel slices for each of the three stages of development were critically aligned according to the marker DNA positions shown, and the slice position was plotted on the abscissa of the graph; the position having the maximum counts for each developmental stage was plotted on the ordinate of the graph as having 1.0 relative counts in order to allow comparison of the shapes of the curves. *Strongylocentrotus purpuratus* embryos were grown in the absence or presence of 50 μg of BrdU per milliliter of seawater (*6*), labeled continuously after fertilization with $H_3{}^{32}PO_4$ (< 0.1 μCi per milliliter of seawater), their DNA's were isolated (*15*), and hairpin DNA was prepared by three rounds of denaturation, renaturation, and collection on hydroxyapatite as described (*1, 9*). Nuclease treatment of the hairpin DNA was as described (*1, 16*). The *A. oryzae* S1 nuclease (*17*) used (*16, 18*) was shown not to break duplex φ80 or λ bacteriophage DNA under the same conditions used on the hairpin DNA's. The DNA's were subjected to electrophoresis with tris-EDTA-borate (*19*) as running buffer. ○, Morula; ●, blastula; △, gastrula. (a) Embryos cultured without BrdU. (b) Embryos cultured with BrdU. Fig. 2 (right). Distribution of sizes of sea urchin hairpin [^{3}H-methyl], [^{32}P]DNA after treatment with S1 nuclease. Experimental details were as described in Fig. 1, except that the embryos were continuously cultured in the presence of L-[^{3}H-methyl]methionine (10.7 Ci/mmole, 1.0 μCi/ml) and $H_3{}^{32}PO_4$ (<0.1 μCi/ml). ○, ^{3}H; ●, ^{32}P. (a and d) Morula; (b and e) blastula; (c and f) gastrula; (a to c) embryos grown without BrdU; (d to f) embryos grown with BrdU.

bels sea urchin DNA during embryogenesis by addition of ^{3}H-labeled methyl groups to those cytosine bases that are normally methylated after DNA replication (*13*). We found that the distribution of lengths as well as the predominant length of the methylated hairpin S1 pieces (^{3}H-labeled) shifts to a greater extent during early embryogenesis than does the general population of S1 pieces (^{32}P-labeled) (*1*) (Fig. 2, a–c). When the distribution of lengths of the methylated hairpin S1 pieces derived from the DNA's of embryos raised in BrdU was examined, we found that the normal developmental stage-specific shift in the distribution of the methylated S1 piece lengths did not occur (Fig. 2, d–f).

The observed shifts in the lengths of S1 pieces during normal embryogenesis might be attributed to effects other than sequence changes in vivo within inverted repeats. However, it is not likely that these shifts are a result of an increase during embryogenesis in S1-sensitive interstrand cross-link position in the DNA, of variability in the proportion of bimolecularly reassociated strands in the hairpin preparations, of amplification or deletion in vivo of a portion of the inverted repeats, of stage-dependent loss of genome components during isolation of bulk DNA's, of greater strand breakage in older embryos as a result (for instance) of greater ^{32}P incorporation with the ensuing higher rate of DNA strand breakage, or preference by S1 nuclease for DNA sites modified in some way during embryogenesis. Arguments against this list of possibilities for interpretation of the reason or reasons for the shift in lengths of S1 pieces have been given (*1*), but the pertinent facts are as follows. (i) The distribution of the lengths of exonuclease VII pieces derived from hairpins is constant during early embryogenesis. (ii) The mean (± range) yield of exonuclease VII pieces *or* S1 nuclease pieces derived from hairpins is constant at 3.0 ± 0.23 percent of the genome for three DNA preparations from each of three stages of embryos. (iii) For hairpins double-labeled in vivo, a minority population of S1 pieces (^{3}H-methyl-labeled S1 pieces) shifts in length to a greater degree during embryogenesis than the general population (^{32}P-labeled S1 pieces), and the distribution of ^{3}H- and ^{32}P-labeled S1 pieces is different on the same electrophoretic gel and varies for each gel with respect to developmental stage (Fig. 2, a–c). (iv) If interstrand cross-linked sites were S1-sensitive and increasing in number during embryogenesis, cross-linked DNA would be increasing the yield of hairpin DNA obtained from progressively older embryos [contrary to both (i) and (ii) above] and preferential methylation of cross-linked pieces of DNA would be required in order to satisfy the data shown in Fig. 2, a–c. In addition, it would be required that during early embryogenesis BrdU would prevent any increase in the hypothetical S1-sensitive cross-linkage, in order to satisfy the data shown in Fig. 2, d–f. (v) Since BrdU prevents the normal shift in sizes of hairpin-derived S1 pieces during embryogenesis (Fig. 2, d–f), it would appear that S1 nuclease cuts hairpin stems at base-pair mismatch sites without regard for the degree of methylation of the DNA. In this regard, BrdU apparently does not interfere with the rate of methylation of DNA in vivo since exposure of embryos to 50 μg of BrdU per milliliter of seawater medium does not change the rate of labeling of embryo DNA with [^{3}H]methionine during the preblastula period of development (*11*). Another but less compelling argument against S1 nuclease having a preference for modified DNA is that the distributions of lengths of S1 pieces for early and late stage embryos are not altered when the degree of nuclease activity is varied over a 20-fold range (*1*).

Previous data (*1*) and that shown in Figs. 1a and 2, a–c, suggest that there are sequence changes within inverted repeat DNA sequences during normal embryogenesis. The increase with further embryogenesis in the number of S1 sites on hairpins formed in vitro suggests that a significant portion of the inverted repeats in vivo are becoming less perfect palindromes as development progresses. Figures 1b and 2, d–f, show that during embryogenesis BrdU stops the proliferation of S1 sites on the derived hairpins. Alternative hypothetical mechanisms have been suggested to account for sequence changes within inverted repeats (*1*); these alternatives fall into two categories: (i) there are translocations of sequences into or within inverted repeats or (ii) hypermutation occurs within inverted repeat regions (or both). Because of the magnitude of the number of sequence change events per unit time that must occur to account for the shift in the distribution of lengths of S1 pieces during early embryogenesis [a minimum of 10^{4} events per haploid genome per cell division for the period of development between morula and gastrula stages (*1*)], the ability of BrdU to inhibit such events, and the necessary specificity of a hypermutation mechanism in sparing nondifferentiating informational regions of the genome, we favor translocation rather than mutation as an explanation for the shift in size of the S1 pieces.

These data would be reasonably accommodated if translocations (including inversions) of genomic sequences resulted after the occurrence of pairs of double-strand breaks in genomic DNA; each break should occur at or near a site of twofold axis of symmetry created by a pair of adjacent inverted repeat sequences. Breakage or reunion events (or both) should be prevented by incorporation of BrdU at or near the sites of symmetry. Our data also suggest that methylation of cytosines within 5′CpG3′ dinucleotide sequences (*13*) regulates breakage or reunion (or both). If these latter processes involve sequence-specific enzymes, then replacement with BrdU of thymidines near sites of symmetry containing 5′CpG3′ sequences would prevent the recombination and finally the differentiation events. The data of Evans and Gross (*4*), that development of *Arbacia punctulata* embryos is not affected by BrdU, could be explained if these enzyme recognition sequences exist and in *A. punctulata* do not contain thymidine residues.

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