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Initiation and Suppression of Apical Hairs of *Fucus* Embryos

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INTRODUCTION

Information about control mechanisms in plant embryo development is rather meager. A serious problem for experimental studies lies in the fact that embryos of higher plants are neither free-living nor easy to isolate from their surrounding maternal tissue. They can be cultured *in vitro* only after having attained a certain, usually rather advanced, developmental stage (Raghavan and Torrey, 1963). Although the production of embryoids from undifferentiated cultured callus tissue was achieved almost a decade ago (Reinert, 1959; Steward *et al.*, 1964; Halperin and Wetherell, 1964) and embryoids can now be produced in large numbers from a variety of different plant species by manipulating the chemical environment, such development is quite asynchronous and there remains great uncertainty about the resemblance between the development and control mechanisms of embryoids and those of true embryos.

For experimental studies on plant embryogenesis, *Fucus vesiculosus* L., a littoral marine brown alga, has the advantage of producing large numbers of free-living embryos and being dioecious. Male plants produce spermatozoids in conceptacles which are borne in specialized fronds, the receptacles. The spermatozoids are extruded from the conceptacles following a period of exposure of the thallus during low tide. Oogonia, each with 8 eggs, are likewise produced in conceptacles, but on female plants. The oogonia are shed into the ocean during the incoming tide, and fertilization takes place after egg release from the oogonia. *In vitro* fertilization of *F. vesiculosus* is a simple procedure (Peterson and Torrey, 1968).

Several early authors (e.g., Thuret, 1854; Rostafinski, 1876; Farmer

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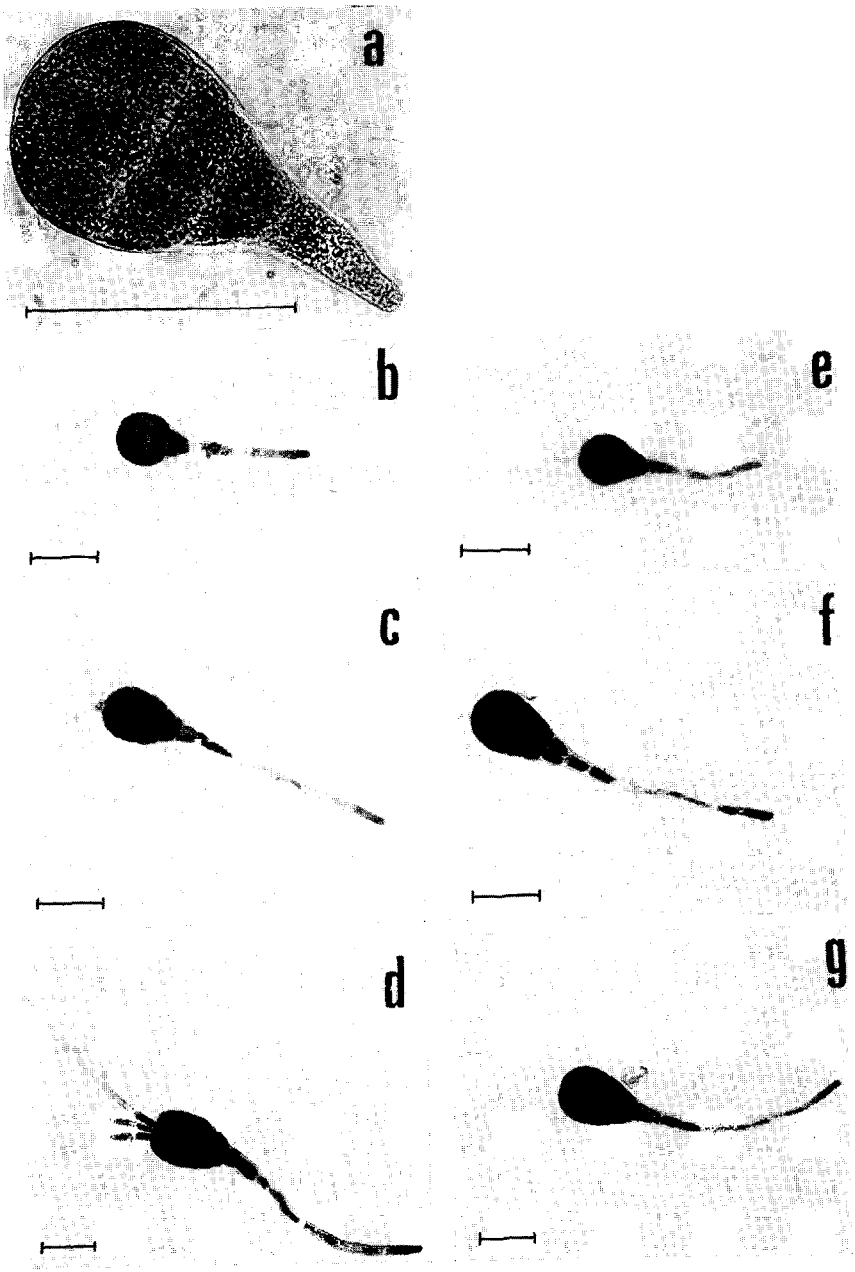

FIG. 1. Stages in the development of *Fucus* embryos. Embryos were fixed in 10% formalin in seawater (SW) after 2, 4, 6, or 8 days in continuous light. (a) 2 days in SW; (b) 4 days in SW; (c) 6 days in SW; (d) 8 days in SW; (e) 2 days in SW followed by 2 days in FU (2×10^{-5} *M*); (f) 2 days in SW followed by 3 days in FU and 1 day in uracil (5×10^{-4} *M*); (g) 2 days in SW followed by 3 days in FU and 3 days in uracil. The scale marker represents 100 μ.

and Williams, 1898; Nienburg, 1929) described the development of *F. vesiculosus* embryos. These descriptions, which were summarized by Nienburg (1931), although given without a timetable, fit well with our own. This normal developmental sequence is illustrated in Fig. 1, a–d. In summary, a protuberance occurs at one end of the initially spherical fertilized egg, causing a polarization of the embryo into rhizoid (at the pointed end) and embryonal "head." Unidirectional light induces the rhizoid to develop away from the light source (see review of Jaffe, 1968). Other physical and chemical gradients also may act to polarize the embryo (Whitaker, 1940). The first mitosis occurs and a cross wall is formed within 24 hours after fertilization. Usually, two more cell walls parallel and on each side of the first one are formed during the next day (Fig. 1a). Therefore, average cell size is progressively reduced during early embryogenesis. In the "head" end, anticlinal and periclinal walls as well as more septa parallel to the original ones, shape the embryo into an elongate pearlike form which at the 3-day stage has a long narrow multicellular rhizoid and a head with more or less isodiametric peripheral and central cells amounting to 20 or more. Subsequently, more anticlinal divisions occur in the peripheral cells accompanied by cell enlargement, but without further segmentation of the inner cells. On day 7 or 8 under 12-hour light (or on day 6 under continuous illumination), one of the large inner cells grows out to produce the first hair (Fig. 1c). This hair is pushed out by an intercalary meristem at its base. Thereafter, more hairs may be initiated in a similar manner (Fig. 1d). One of the basal cells of one of these hairs ultimately becomes the apical meristematic cell of the future plant, giving rise to the meristem within the apical groove of the developing vegetative thallus. Apical hair initiation is therefore an obligatory stage in *Fucus* development, crucial to the subsequent development of the thallus. In the present report, experimental studies on this stage of embryo development are presented.

MATERIAL AND METHODS

Male and female plants of *Fucus vesiculosus* L. were collected from the intertidal rocky areas of Bass Rocks, Gloucester, Massachusetts, between November, 1967 and July, 1968. The gametes were obtained and fertilized as previously reported (Peterson and Torrey, 1968) with the following modifications. Whenever a 1-day exposure of male receptacles to 15°C did not result in adequate extrusion of sperm from the conceptacles, they were placed for an additional day in refrigeration (0–4°C).

Eggs were obtained by placing female receptacles in seawater, as described previously, but during the spring and summer months egg release could be improved by keeping receptacles overnight at 0–4°C rather than for a few hours at 15°C. The latter procedure occasionally resulted in abundant slime release, and then several washings of the eggs by repeated agitation with fresh seawater were required in order to assure good fertilization. A delay of more than 45 minutes from the beginning of fertilization to the washing of the fertilized eggs over a 35 μ mesh Nitex filter resulted in clumping of the young embryos as well as sticking of masses of sperm to these embryos. This occurred because the fertilized eggs rapidly form a wall and become sticky at about this time after fertilization. The fertilized and washed eggs were transferred to filtered (Millipore, HAWP, 0.45 μ pore size) seawater which contained 25 μg of streptomycin and 50 units of penicillin per milliliter. It was found in preliminary tests that this antibiotic solution did not affect growth and development of the *Fucus* embryos but effectively eliminated bacterial contamination within 12 to 16 hours (at 15°C), as tested by plating embryo suspensions on agar medium supporting marine bacteria (Carlucci and Pramer, 1957). The required number of fertilized eggs per replication was obtained by counting an aliquot and subsequent dilutions. All but the incorporation experiments were performed in 6-cm diameter plastic petri dishes. The young embryos stick to the bottom of the dishes, thus enabling easy exchange of media. The media were exchanged every 2 or 3 days, and the embryos were kept continuously at 15°C with 12 hours of fluorescent light per 24-hour period.

RESULTS

When *Fucus* embryos were cultured under conditions described above they showed a remarkable uniformity of growth and development. Over 95% of the embryos initiated rhizoids 16–20 hours after fertilization. Apical hairs appeared in 50% or more of the plants after 8 days of culture in a 12-hour (fluorescent) light regime, and 90% or more had apical hairs after one more day. Under continuous light, even greater uniformity of apical hair formation was achieved, and apical hairs appeared at 6 days after fertilization in most of the embryos.

6-Methylpurine and 6-azauracil are known to be potent inhibitors of RNA synthesis and rather effective on plant systems. We found that they are probably useless for the study of *Fucus* development because their effect on differentiation was nonspecific. When used at 10^{-5} to

10^{-3} *M* they progressively retarded growth and development. Still when embryos were cultured in 10^{-3} *M* 6-azauracil, rhizoids did develop and apical hairs appeared at 12 days even though at this time these embryos were rather smaller than control embryos prior to hair initiation.

The specificity, in respect to hair initiation of a third base analog, 8-azaguanine, is also doubtful. It had no apparent effect up to 10^{-7} *M*, retarded hair formation but not growth at 3×10^{-7} *M* and strongly inhibited both growth and hair formation at 10^{-6} *M*. Moreover, the highest concentration of guanine which could be solubilized in seawater (10^{-5} *M*) only partially reversed the 8-azaguanine effect.

When 5-fluorouracil (FU) was applied to the medium of cultured embryos, it showed no apparent effect over a rather broad range of concentration (10^{-5} to 10^{-3} *M*) during the first 6 days after fertilization. Thereafter, rhizoid and "head" growth continued, but apical hair forma-

TABLE 1

EMBRYO ELONGATION AND APICAL HAIR FORMATION AS AFFECTED BY 5-FLUOROURACIL, URACIL, AND THYMIDINE[a]

Treatment	Embryo age						
				8 Days		11 Days	
	2 Days, length[b]	4 Days, length	6 Days, length	Length	Number of apical hairs	Length	Number of apical hairs
Seawater control	175 ± 2[c]	349 ± 2	485 ± 4	590 ± 5	18/36	751 ± 12	28/30
Uracil (5×10^{-4} *M*)	180 ± 2	348 ± 2	496 ± 4	629 ± 6	24/36	784 ± 11	29/30
Thymidine (5×10^{-4} *M*)	182 ± 2	353 ± 4	506 ± 5	610 ± 6	18/36	777 ± 14	29/30
5-Fluorouracil (2×10^{-5} M)	173 ± 1	336 ± 5	485 ± 10	554 ± 7	0/30	678 ± 12	0/30
5-Fluorouracil and uracil	182 ± 2	356 ± 3	505 ± 5	635 ± 8	17/30	797 ± 8	28/30
5-Fluorouracil and thymidine	182 ± 2	347 ± 3	480 ± 4	542 ± 4	0/30	724 ± 10	0/30

[a] Embryos were exposed continuously to treatment solutions and given 12 hours of light daily; 3 petri dishes with several hundred embryos in each were used per treatment; measurements were made at random on 10 or 12 plants per dish.

[b] In microns.

[c] $\pm\text{SE}\left(\pm\sqrt{\frac{d^2}{n(n-1)}}\right)$.

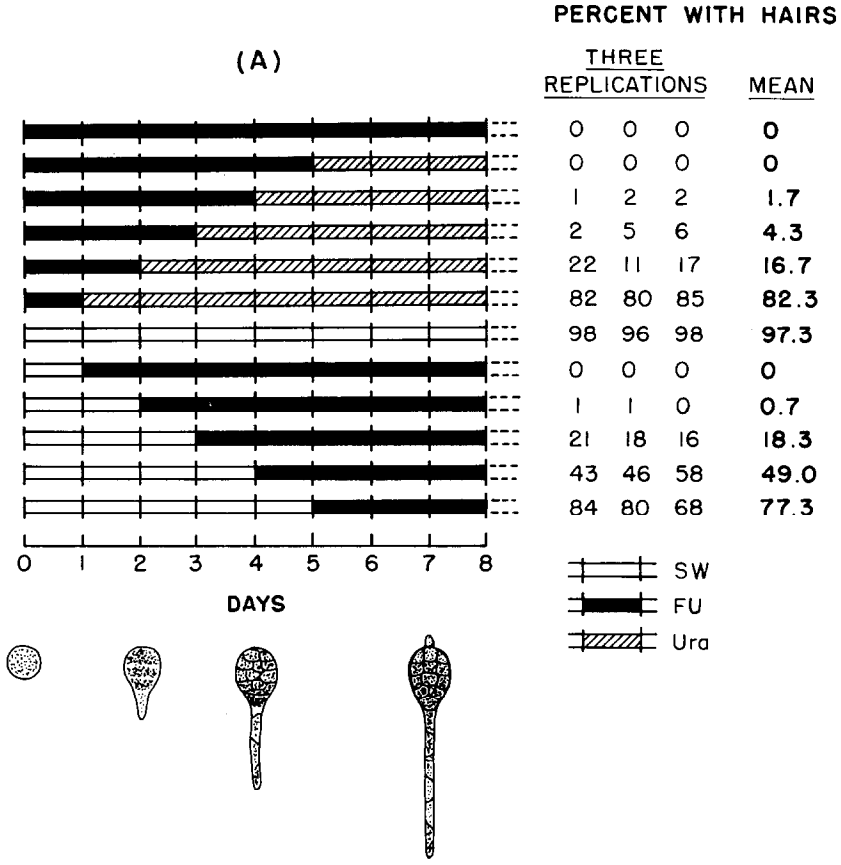

FIG. 2. Formation of apical hairs in the absence and presence of FU. Embryos were cultured in 12 hours light daily, in seawater, in FU (2×10^{-5} *M* and 5×10^{-4} *M* thymidine) or in uracil (5×10^{-4} *M*) as indicated; 3 petri dishes with several hundred embryos in each were used per treatment; observations were made at random on 100 embryos per dish 2 weeks after fertilization. (A) Effect of "early" or "late" FU exposure on apical hair formation. (B) Effect of 1-, 2-, or 3-day exposure to FU on apical hair formation.

tion was *completely* eliminated. Results of an experiment in which several controls were included are summarized in Table 1. FU alone as well as FU in the presence of thymidine prevented apical hair formation while uracil and thymidine when used separately had no effect on hair initiation and only slightly promoted growth after extended culture. It is obvious from Table 1 that after 11 days the hairless FU embryos

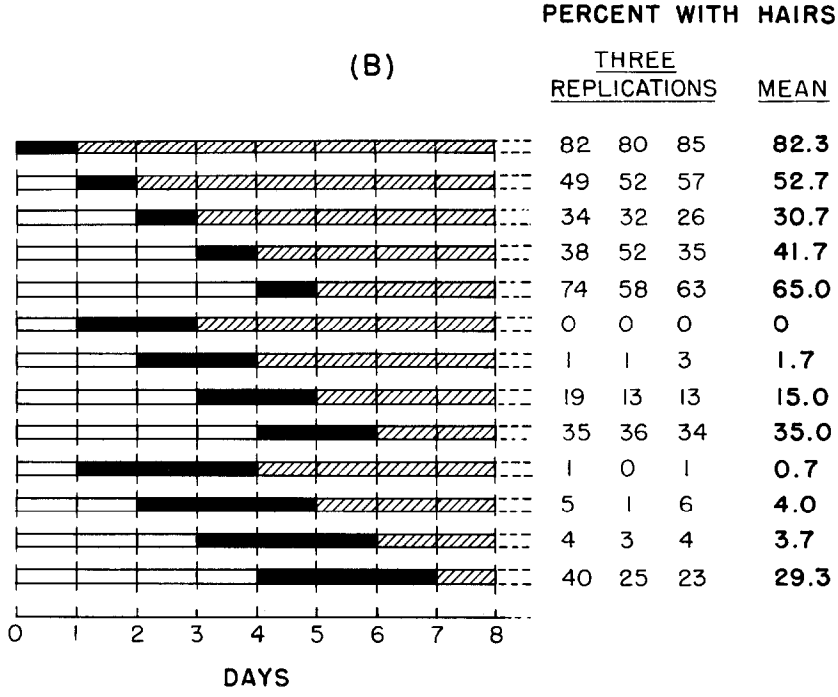

FIG. 2B

and, even more so, the embryos with FU in the presence of thymidine had exceeded in size the 8-day-old control embryos which at that time already had hairs. Moreover, it should be pointed out that growth and cell multiplication did not stop in the embryos treated with FU or FU plus thymidine even after 11 days, so that after 3 weeks' culture the treated embryos far exceeded the controls at time of hair initiation. In fact, extension of the rhizoid and extension and cell multiplication in the "head" (as observed externally on the peripheral cells) continued indefinitely in the presence of FU. Still no hairs were ever seen in them. Figure 1 shows embryos cultured either continuously in seawater or transferred after 2–3 days to FU and then allowed to recover in uracil.

We wanted to know for how long after fertilization the FU treatment must proceed in order still to be effective in inhibiting apical hair formation and, on the other hand, how late after fertilization FU treatment is still effective. Treatments were thus given as shown in Fig. 2A. It was found that only a few (13 out of 300) embryos produced apical

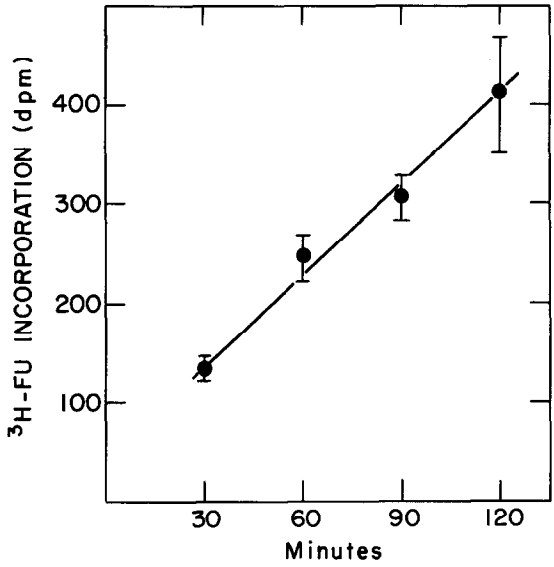

FIG. 3. Incorporation of ^{3}H-FU into *Fucus* embryos. Three-day-old embryos, cultured in glass vials, were incubated with 10 μC/ml ^{3}H-6-FU of 2×10^{-5} *M*. At the end of incubation, embryos were handled as described in Table 2, but the Millipore filters were counted by liquid scintillation counting. Triplicate samples of 8000 embryos each served for each incubation time. Incorporation values represent dpm/8 $\times$ 10^3 embryos.

hairs when cultured for the first 3 days in FU and allowed to recover in uracil. Longer exposures to FU practically prevented hair formation. On the other hand even continuous culture in FU, starting 2 or 3 days before the beginning of hair formation, did not prevent the appearance of hairs in most or in half, respectively, of the embryos. When treated for the first day only, there was almost no retardation of apical hairs. Therefore it seemed that days 2, 3, and 4 were the decisive period even though hairs became visible in this experiment only on day 7. In order to obtain more information on this point embryos were exposed to FU for only a limited number of days and then transferred to uracil (Fig. 2B). It was observed that all treatments covering the third day were the most effective. From preliminary anatomical observations of embryos cultured in continuous light and having hair initials after 6 days, we know that at the 4-day stage no internal indication of hair differentiation is yet observable.

The incorporation of FU into the tissues of higher plants is known

TABLE 2

INCORPORATION OF LEUCINE-^{14}C AND ADENINE-^{14}C INTO *Fucus* EMBRYOS IN THE PRESENCE AND ABSENCE OF 5-FLUOROURACIL (FU)[a]

Treatment	Counts per minute per 5 × 10^3 embryos	
	Leucine-^{14}C	Adenine-^{14}C
Without FU	264	810
	203	928
	274	910
With FU	215	1090
	237	843
	187	851

[a] One-day-old embryos cultured in glass vials were incubated for 60 minutes in either 0.5 μC/ml leucine-^{14}C, g.l., 0.2 mC/mmole, or in 0.5 μC/ml adenine-8-^{14}C, 52 mC/mmole in seawater (SW), with or without 2 × 10^{-5} *M* FU. After incubation the embryos were thoroughly washed with SW, then fixed and retained overnight in cold 7% TCA. They were then homogenized in cold TCA and washed over Millipore filters with additional 6 × 1.5 ml TCA. The filters were glued on planchets and counted with a low background gasflow counter.

(Bonner and Zeevaart, 1962; Key, 1966) but has not yet been reported for brown algae. As is evident from Fig. 3, FU is incorporated into *Fucus* embryos. The incorporation into 3-day-old embryos is approximately linear with time. When embryos with incorporated FU-^{3}H were fixed, homogenized, washed with 7 % cold trichloroacetic acid, incubated overnight at 37°C with 0.3 *N* KOH and reprecipitated with perchloric acid in the presence of carrier yeast RNA, it was found that all the incorporated FU-^{3}H was KOH hydrolyzable. It is thus assumed to be incorporated into RNA.

FU did not affect leucine incorporation and only slightly reduced adenine incorporation into *Fucus* embryos (Table 2). Moreover, it was found that both incorporation and uptake of adenine were only slightly affected by FU, and that this 10% inhibition was not increased even when the embryos were kept in FU for 1 day prior to the incorporation test (Table 3). These results suggested that FU had no quantitative effect on either protein or RNA synthesis of *Fucus* embryos.

DISCUSSION

Although *Fucus vesiculosus* L. is obviously a favorable object for the study of embryo development, previous experimental work has been restricted to the very early stages of embryogenesis, especially

the polarization following fertilization (see Jaffe, 1968). The present study is the first, to our knowledge, which deals with a later stage, viz., apical hair formation. It was found in the present study that this critical stage of differentiation, which is obligatory for the future development of the *Fucus* plant, can be "uncoupled" from growth, i.e., no hairs will ever be formed but the embryo goes on with cell division and extension of size. This ability to continue indeterminately a given phase of development without necessarily starting with the next phase, although rather rare in animals, is relatively common in plants. Nonetheless, it had not been previously shown for plant embryos. Thus, in plant systems specific inhibitors can be used to prevent the entry into the next developmental stage (whether induced by internal regulation, e.g., embryogenesis, or induced by external factors, e.g., photoinduction of flowering). But the use of these inhibitors is permissible only if they act specifically on development and are not merely causing a general retardation of growth (Miller, 1968). The effect of FU on apical hair initiation was shown in this study to be rather specific, since hairs were eliminated while growth continued.

It should be noted that the strongest inhibitory effect of a short FU application was on 3-day-old embryos. Thus, this effect preceded by at least 2 days the visible cellular changes which bring about hair formation. Although we do not fully understand the mode of action of FU, this base analog certainly interferes with the synthesis of certain forms of RNA (see review of Heidelberger, 1965). We deduce, therefore, that the specific RNA needed for apical hair initiation was not present in a "masked" form (Spirin, 1966) in the *Fucus* egg, but rather was transcribed in the young embryo, probably between the second and fourth day. The lack of FU effect when applied only during the first day may be interpreted to mean that at this time the RNA involved in apical hairs was not yet transcribed, yet it might also be explained by the relatively low incorporation of externally applied RNA precursors into 1-day-old embryos (Koehler and Linskens, 1967; unpublished results of the present authors). The strong reduction in the FU effectivity after the fourth day probably shows that at this stage the transcription of the specific RNA needed for hair initiation was already terminated.

FU showed no significant quantitative reduction of incorporation of protein or nucleic acid precursors into *Fucus* embryos. We assume that its effect on morphogenesis is mediated through a qualitative rather than a quantitative change of RNA. Of particular interest is the question of how FU allows continuous growth, hence also continuous production of necessary proteins, but prevents the shift into the next phase

TABLE 3

INCORPORATION AND UPTAKE OF ADENINE-^{14}C INTO *Fucus* EMBRYOS IN THE PRESENCE AND ABSENCE OF 5-FLUOROURACIL (FU)[a]

Treatment	Counts per minute per 2×10^3 embryos			
	Incorporation		Uptake	
	No FU	With FU	No FU	With FU
1-hour incubation	5019	4892	13550	10070
	4805	3979	19160	20925
2-hour incubation	12466	10726	25990	29305
	11488	12561	34280	39480
3-hour incubation	14636	18581	42520	46845
	17145	17685	40095	44830
4-hour incubation	27712	24024	64105	59090
	28446	24097	73205	55535
1-day FU preincubation	—	18606	—	53290
and 3-hour incubation	—	16776	—	51695
in FU	—	—	—	—

[a] Three-day-old embryos, cultured in glass vials, were incubated in 1.5 μCi/ml of adenine-8-^{14}C, 52 mC/mmole. FU concentration was 2×10^{-5} *M*. Incorporation was determined as in Table 2. Uptake represents the TCA-soluble counts of the same embryos.

of differentiation. Before trying to speculate on this problem, one should recall that FU inhibits the induced synthesis of β-galactosidase in *Escherichia coli* while allowing, at the same time, the synthesis of constitutive enzymes (Horowitz and Chargaff, 1959). FU completely prevents photoinduced sporulation in the fungus *Trichoderma* while vegetative growth continues indefinitely (Galun and Gressel, 1966). In higher plants the effect of FU is in some cases problematic (see Rimon and Galun, 1967) but FU obviously inhibits floral induction in *Xanthium* (Salisbury and Bonner, 1960; Bonner and Zeevaart, 1962). In these three cases FU interfered with the regulation of induction while not (or barely) affecting the established metabolic or cellular activity. In the regulation of gene expression in such systems regulatory proteins may have a prime role. Furthermore, they may exert their role by an allosteric mechanism. If this is the case, then a slight change in amino acid sequence causing a steric shift may render them inactive as regulators.

SUMMARY

Eggs of *Fucus vesiculosus* L. were fertilized and cultured *in vitro*, and attention was focused on the formation of apical hairs. Each of

these hairs is an outgrowth of one of the enlarged internal cells of the embryonal "head." One of the basal hair cells is converted into the apical meristematic cell from which the entire future plant develops. Thus, apical hair formation plays a primary role in morphogenesis in *Fucus*.

Apical hairs appear in most cultured embryos 6 days after fertilization (under continuous illumination, and 1–2 days later under 12 hours light). When cultured in seawater supplemented with 10^{-5} to 10^{-3} *M* 5-fluorouracil (FU) growth proceeded at almost the normal rate and continued indefinitely, but no apical hairs were ever found. Thymidine and uracil, applied separately in seawater, had no effect on hair formation. Uracil, but not thymidine, counteracted the effect of FU. A 2-day application of FU, followed by recovery in uracil, prevented hair formation, provided the 2-day treatment period included day 3 after fertilization. Application of FU after day 4, even given continuously, had only a slight effect on hair formation or no effect at all. These and other results showed that FU exerted its strongest effect at least 2 days before any cellular indication of hair initiation. Using FU-^{3}H as the inhibitor, it could be demonstrated that FU is incorporated into a KOH-hydrolyzable fraction presumed to be RNA. It is speculated that the RNA involved in hair initiation is transcribed between 2 and 4 days after fertilization, and that the specific effect of FU is based on the involvement of regulatory allosteric proteins which are rendered inactive with even slight mistakes in configuration mediated through the falsification of RNA by FU, while enzymes and other nonregulatory proteins are less affected by FU and retain their function.

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