A Quantitative Bioassay for HIV-1 Based on Trans-Activation

BARBARA K. FELBER AND GEORGE N. PAVLAKIS

A bioassay that is based on trans-activation has been developed for the detection and quantitation of the human immunodeficiency virus type 1 (HIV-1). Indicator cell lines were constructed that contain the HIV-1 long terminal repeat ligated to the chloramphenicol acetyltransferase (CAT) gene. Infection of these cells by HIV activates the expression of CAT protein. Isolates of HIV-1 with divergent nucleotide sequences activated the indicator cell lines to a similar extent, approximately 500- to 1000-fold. Human T cell lymphotropic viruses types 1 and 2, equine infectious anemia virus, and herpes simplex virus 1 did not activate the indicator cell lines. Isolates of simian immunodeficiency virus and human T cell lymphotropic virus type 4 activated these cells to a much lesser extent, which suggests that these viruses contain similar, but distinct, trans-activators. This assay can be used for the detection, quantitation, and typing of HIV and for studying the effect of drugs on the replication of HIV in different cellular backgrounds.

THE EXPRESSION OF THE HUMAN IMmunodeficiency virus type 1 (HIV-1) (*1*) is greatly increased through the concerted function of two viral activator proteins, the trans-activator or *tat* protein (*2–6*) and the *art* or *trs* protein (*7, 8*). The trans-activator is a 14-kD protein of 86 amino acids. It interacts directly or indirectly with sequences within the R region of the long terminal repeat (LTR) of HIV-1 and increases the steady-state levels of viral messenger RNA (mRNA) (*4–6*). The HIV-1 trans-activator is specific in its action in different cell lines (*4*). In human cells, the levels of mRNA increase approximately 10 times, whereas the amount of protein produced from this mRNA increases more than 500 times (*4, 5*). Therefore, in human cells the trans-activator increases, directly or indirectly, the utilization of this mRNA (*4, 5*). In some animal cells the increase in protein has been attributed to mRNA induction or stabilization (*6*).

We have constructed human indicator lymphoid cell lines that contain integrated copies of HIV-1 LTR ligated to the chloramphenicol acetyltransferase (CAT) gene and that can be infected by several isolates of HIV. The LTR-CAT constructs retain their inducibility by *tat* when integrated stably into the genome of mammalian cells (*4*). Upon infection of the indicator cells by HIV, *tat* protein is produced, and the expression of CAT protein is increased by more than 500 times. Such cell lines were used to detect as few as ten HIV-infected lymphoid cells in 2 days and to quantitate the effects of antivirus substances on infected indicator cells.

National Cancer Institute, Frederick Cancer Research Facility, Bionetics Research, Inc., Basic Research Program, P.O. Box B, Frederick, MD 21701.

To introduce LTR-CAT constructs into human lymphoid cells, we constructed two recombinant retroviral vectors described in Fig. 1. The integrated proviruses are expected to have two internal promoters, the SV40 promoter and the HIV-1 promoter flanked by the murine sarcoma virus (MSV) LTRs. Recombinant virus stocks were generated (*9–11*) and were used for the infection of several human cell lines including H9, U937, and Molt4. All of these cell lines contain the T4 receptor, which is necessary for infection by the HIV-1 virus (*12*). After infection by the recombinant retroviruses, G418-resistant cells were isolated and assayed for the presence of CAT enzyme before and after infection by HIV-1. More than 90% of the G418-resistant cell lines produced high levels of CAT enzyme upon infection by HIV-1, whereas the uninfected cells produced very low levels of enzyme. On the basis of these results, several cell lines (H938, H9317, H9318, M311, and U38) were selected for further evaluation. The cell lines were assayed for the presence of the CD4 molecule by fluorescence-activated cell sorting (FACS).

To verify that the HIV-1 promoter was correctly regulated by *tat* when integrated within the MSV LTRs, we quantitated the correctly initiated mRNA by S1 mapping (Fig. 2) and the protein that was produced by CAT assays (Fig. 3). Quantitation of the mRNA levels before and after infection of H938 cells indicated that after infection by HIV-1, five to ten times as much mRNA (initiated at the cap site) was present in the lymphoid cells (Fig. 2). When the H938 cells were infected by simian immunodeficiency virus (SIV), the mRNA increase was lower (two to three times, lane 2). Analysis of the mRNA by Northern blots confirmed the conclusion of the S1 experiments. The observed increase in CAT mRNA was approximately tenfold, similar to that reported for HeLa cells (*4*). In parallel experiments, the activation of CAT enzyme after infection of H938 cells was greater than 500 times. Cell lines M311 and U38, which are derivatives of Molt4 and U937, respectively, gave similar results. Thus, the activation of HIV-1 LTR after viral infection was similar to that reported for other human cells (*3–5*). Integration via the recombinant retroviral vector did not affect the activation by *tat*. The increase in mRNA also occurred in cell lines H9317 (Fig. 2, lanes 5 and 6) and H9318 (lanes 7 and 8), which contain integrated GVL3CATa (Fig. 1). Therefore, both orientations of LTR-CAT in the retroviral vectors can be activated after HIV-1 infection. That the activation of the viral LTR was dependent on the HIV-1 trans-

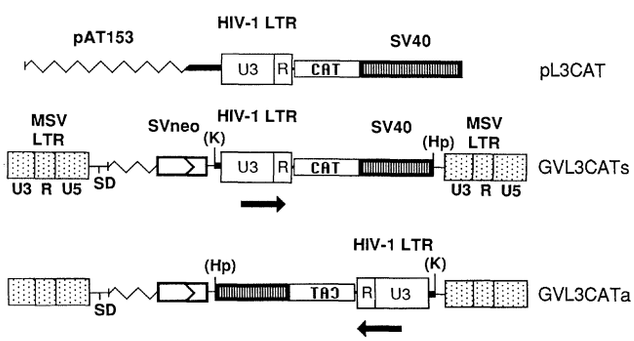

Fig. 1. Recombinant constructs. Plasmid pL3CAT has been described elsewhere (*4*). GVL3CATs and GVL3CATa are shown in the proviral form, containing two MSV LTRs. Plasmids pGVL3CATs and pGVL3CATa contain one MSV LTR. In order to construct pGVL3CATs and pGVL3CATa, we introduced a Kpn I–Hpa I fragment of pL3CAT containing the HIV-1 LTR (nucleotides −524 to +80, where +1 is the transcriptional start), the structural gene for chloramphenicol acetyltransferase (CAT) and SV40 sequences, including the small t splice region, into the retroviral vector pGV1 (*25*) in the sense (s) and antisense (a) orientation relative to the LTR of the vector. Symbols are: dotted box, murine sarcoma virus HT-1 (MSV) LTR; thin line, viral packaging signal and splice donor (SD); wavy line, pBR322 origin of replication; open box, SV40 origin of replication and early promoter ligated to the *neo* gene which confers resistance to neomycin in bacteria and to antibiotic G418 in animal cells; CAT, chloramphenicol acetyltransferase gene; striped box, SV40 sequences including the small t splice region. Arrows indicate the transcriptional orientation of the HIV-1 LTR-CAT. Hp, Hpa I; K, Kpn I.

activator was verified by fusing the indicator cell lines to cells such as CB2MX3 (*4*), which express *tat* in the absence of any other HIV proteins. The results indicated (*13*) that the activation of the LTR-CAT after infection was caused by the *tat* protein and no other viral protein was required.

By using HIV_{RF}, a Haitian isolate that is very divergent in nucleotide sequence from the prototype HIV_{IIIB} (*14*), we have shown that both cell-free virus (RF medium) and infected cells (RF cells) could activate CAT expression of H938 cells (Fig. 3). However, cocultivation was the more efficient method of virus transmission. Other isolates of HIV-1 that differ extensively in nucleotide sequence, such as HIV_{IIIB}, HIV_{MN}, and HIV_{HXB2}, activated the indicator cells to a similar extent. The induction of CAT in the indicator cell line is a function of the amount of HIV virus (or of the infected cells) and of the incubation time. The amount of CAT enzyme produced after infection of the indicator cells was quantitated by calculating the initial slopes of the CAT reactions and comparing them to known quantities of purified CAT enzyme (*15*). There is a linear relation between the amount of infecting virus and the produced CAT enzyme within at least two orders of magnitude when the indicator cells are in excess (Fig. 3B). In its present form, the assay can detect about ten HIV-producing cells, or virus from 10^3 infected cells, after 2 to 3 days in culture.

Several other viruses were tested in this assay. Human T cell lymphotropic virus types 1 and 2 (HTLV-I and HTLV-II), equine infectious anemia virus (EIAV), and herpes simplex virus 1 (HSV-1) were not able to activate the indicator cell lines after infection (*16*). These results were verified with a different assay in which cells productively infected with either HTLV-1 or EIAV were fused to H938 cells using 50% polyethylene glycol. No activation of CAT production was detected. Isolates of SIV (*17*) and HTLV-IV (*18*) were able to activate CAT expression in the indicator cells, but the maximum activation under saturating virus conditions was approximately 40-fold (average of three independent experiments), that is, roughly one order of magnitude less than HIV-1. The induction by SIV was also much lower at the mRNA level (Fig. 2, lane 2). These results suggest that the *tat* genes of these viruses are similar but not identical to the HIV-1 *tat*, as previously suggested (*19*). We have not yet determined the extent of activation of these cell lines by HIV-2 (*20*).

HSV-1 did not affect the expression of CAT in H938 cells. It has been reported that HSV-1 activates the expression of HIV-1 LTR (*21*). We have also shown that HL3T1 cells [a HeLa cell line containing integrated copies of pL3CAT (*4*)] are activated to produce CAT after infection by HSV-1 (*13*). This activation is one order of magnitude less than the activation obtained by HIV-1 *tat* (40-fold compared to 500-fold, Table 1). To understand the reason for the discrepancy between the activation of LTR-CAT by HSV-1 in H938 and HL3T1 cells, we also studied the activation of clones HLGVL3Cs-3, HLGVL3Cs-9, and HLGVL3Cs-10 (Table 1), which were gen-

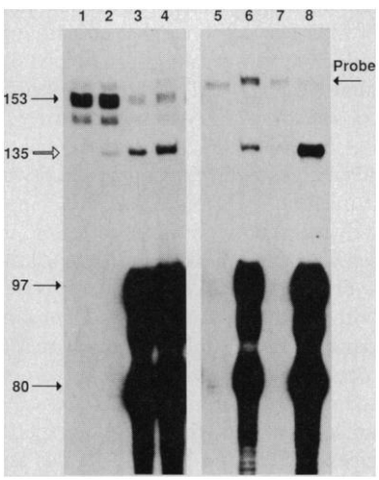

Fig. 2. Nuclease S1 mapping after infection of indicator cells by HIV-1. H938 cells (lanes 1 to 4) were infected by cocultivation with H9/SIV or H9/HIV-1. Cell lines H9317 (lanes 5 and 6) and H9318 (lanes 7 and 8), which contain integrated copies of GVL3CATa, were infected by cocultivation with $H9/HIV_{HXB2}$ cells. The $H9/HIV_{HXB2}$ line was generated by DNA transfection of an infectious molecular clone (*26*). Two to 3 days after infection of the indicator cells total RNA was prepared by the hot phenol procedure, 20 μg of which was hybridized to an excess of a uniformly ^{32}P-labeled single-stranded probe generated as described (*4*). This probe contained 8 nucleotides from the M13 vector, 97 nucleotides from the HIV-1 LTR (nucleotides −17 to +80), and 56 nucleotides from the CAT gene. The hybridization reactions were treated with S1 nuclease and analyzed as described (*24*). Lanes shown are: 1, uninfected H938 cells; 2 to 4, H9318 cells infected with SIV, HIV_{IIIB}, or HIV_{RF}, respectively; 5, uninfected H9317 cells; 6, H9317 cells infected with HIV_{HXB2}; 7, uninfected H9318 cells; 8, H9318 cells infected with HIV_{HXB2}. The position of the undigested probe is indicated to the right. In the infected cells the probe hybridized with both the GVL3CAT mRNAs and the HIV-1 mRNAs. Hybridization to the GVL3CATs mRNAs that initiate upstream of the HIV LTR produces a band of 153 nucleotides after S1 digestion. This band is absent in cell lines H9317 and H9318, which contain integrated copies of GVL3CATa. Hybridization to the correctly initiated LTR-CAT mRNA produces a band of 135 nucleotides. Hybridization to the HIV-1 mRNAs produces bands of 80 and 97 nucleotides from the 5′ and the 3′ untranslated regions, respectively, because of the terminal redundancy.

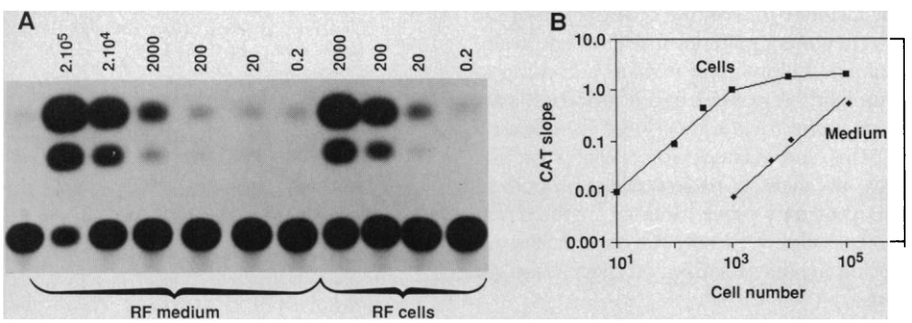

Fig. 3. (**A**) Detection of the HIV_{RF} virus by CAT assay using the H938 cell line: 2×10^5 H938 cells were cocultivated for 2 days with the indicated amounts of infected cells ($H9/HIV_{RF}$) or medium from these infected cells in a total volume of 3 ml. Two days later an aliquot (1/6) of cells was removed, lysed, and assayed for CAT as described (*15*). The first lane represents uninfected H938 cells. The label RF medium indicates infection by cell-free medium, which was collected after 24 hours of cultivation from the number of $H9/HIV_{RF}$ cells indicated at the top and was used to infect 2×10^5 H938 cells. The label RF cells indicates infection by cocultivation. The numbers of $H9/HIV_{RF}$ cells included in the cocultivation are indicated at the top. (**B**) Titration of HIV_{RF} virus after 2 days in culture. The initial slopes of CAT enzymatic reactions were plotted versus the dilution of infected cells or media from infected cells.

Table 1. Activation of different L3CAT constructs in HeLa cells.

Construct	Activation	
	HSV-1 infection*	*tat*†
Stable HeLa cell lines‡		
HL3T1	40×	>500×
HLGVL3Cs-3	1×	>200×
HLGVL3Cs-9	2×	>200×
HLGVL3Cs-10	2×	>200×
Transient expression in HeLa cells§		
pL3CAT	10×	>200×
pGVL3CATs	1×	>200×
pGVL3CATa	1×	>200×

*Ten plaque-forming units of HSV-1 per HeLa cell were applied, and the CAT enzyme was measured after 2 days. †*tat* was provided by fusion of the stable cell lines to CB2MX3 cells using polyethylene glycol or by transfection with the *tat*-producing plasmid pMX3N (*4*) (transient expressions). ‡HL3T1 cells were derived as described (*4*). The three independent HLGVL3Cs cell lines were derived from HeLa cells by GVL3CATs recombinant retroviral integration. §The indicated plasmids were transfected into HeLa cells by calcium phosphate coprecipitation as described (*24*).

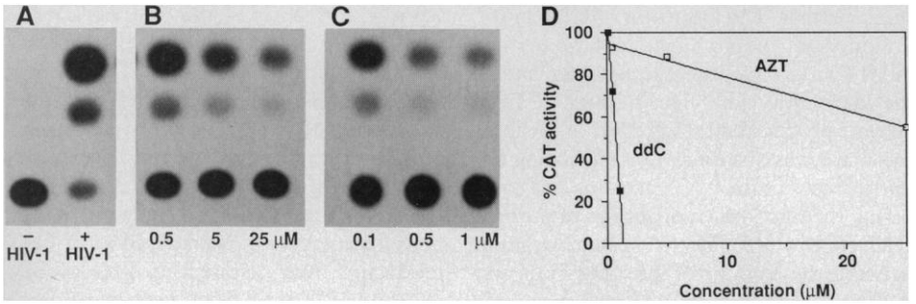

Fig. 4. Inhibition of HIV replication by nucleotide analogs. A 2-day assay was used for the quantitation of HIV replication in H938 cells in the presence of nucleotide analogs AZT or ddC: 10^5 H938 cells were mixed with 10^4 HIV_{RF}-infected H9 cells, and CAT enzyme was measured 48 hours later. (**A**) CAT assays of control cultures incubated in the absence (−HIV) or in the presence (+HIV) of virus (no drug control). The indicated concentrations of AZT (**B**) or ddC (**C**) were added at the time of viral infection. The initial slopes of the CAT enzyme reactions were proportional to the amount of CAT and were expressed as a percentage of CAT activity of the no drug control. (**D**) Obtained CAT values plotted versus the drug concentration.

erated after integration of GVL3CATs in HeLa cells. In addition, the effects of HSV-1 infection on the LTR-CAT were studied by transient expressions. The results of these experiments (Table 1) indicated that HSV-1 infection activated only the expression from pL3CAT and not from GVL3CATs and GVL3CATa. We conclude that activation by HSV-1 is prevented by cis-acting elements in the GVL3CAT constructs. The mechanism of this inhibition is not clear. Viral regulatory proteins have been shown to repress transcription from some promoters (*22*). Whatever the mechanism of this effect, it results in indicator cell lines that are very specific and allows the use of H938 cells for the detection of HIV even in the presence of HSV-1. This property may be important in the use of this assay for the detection of HIV in clinical specimens. These results also suggest that *tat* and HSV-1 infection activate the HIV LTR via different mechanisms.

We then used these indicator cell lines in a 2-day bioassay for the quantitation of drug inhibition of acute infection by HIV-1. Figure 4 demonstrates the effects of two nucleotide analogues on the replication of HIV after infection of H938 cells. We mixed 10^5 H938 cells with 10^4 HIV_{RF}-infected H9 cells and the indicated amounts of 3′-azido-3′-deoxythymidine (AZT) or 2′,3′-dideoxycytidine (ddC). In agreement with previous studies (*23*), both compounds inhibited the replication of HIV. The percent inhibition for each concentration of these compounds was calculated after quantitations of the produced CAT enzyme. It was shown that ddC is 50 times as effective on a molar basis as AZT (Fig. 4D). These results demonstrate that the assay can be used as a rapid and quantitative in vitro screening procedure that allows the evaluation of anti-HIV agents.

The assay we describe here is based on a new concept, that is, the genetic engineering of indicator cell lines that contain a marker inducible by a viral regulatory protein. The same strategy can also be applied to the construction of specific indicator cell lines for other viruses. This assay has certain advantages over existing methods (*1, 23*); namely, it is specific for HIV, it is not influenced by several other viruses, and it is sensitive and relatively rapid. Since the virus is detected by CAT enzyme measurements, both cell-free virus and virus-infected cells can be assayed, unlike methods that rely on measurements of viral protein (reverse transcriptase and immunofluorescence). Also, unlike methods that rely on specific cell types (ATH8 and MT2) (*23*), this assay can be adapted to several different cell types. Therefore, the time course of infection as well as the effect of drugs can be studied quantitatively in different cell lines. Different assay configurations can measure the effect of various drugs on either acute or chronic HIV infection. The establishment of an easy quantitative assay for infectious HIV may facilitate the routine quantitation of the virus in patient material and allow the evaluation and follow-up of patients according to virus load. A panel of indicator cells may prove useful for the functional classification of HIV and related virus isolates on the basis of their trans-activating properties. This assay may prove valuable for the development of new therapeutic approaches, including the ones focusing on specific anti-*tat* drugs.

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Cell Wall Is Required for Fixation of the Embryonic Axis in *Fucus* Zygotes

DARRYL L. KROPF,* BERNARD KLOAREG,† RALPH S. QUATRANO‡

Establishment of a primary developmental axis generally is thought to involve rearrangements in the plasma membrane or cytoplasm of the egg. In this report the additional requirement for cell wall in polarization of *Fucus* zygotes was investigated. Protoplasts of fertilized eggs were tested for their ability to establish an axis in accordance with an orienting vector of unilateral light. The results demonstrate that cell wall is not required for axis formation. However, the orientation of the axis remains labile until new cell wall is synthesized. The presence of a cell wall is an absolute requirement for axis fixation.

FORMATION OF A PRIMARY EMBRYonic axis establishes the framework on which all subsequent development depends, yet it is not well understood. The main difficulty in studying axis formation is that in most organisms it takes place early in the differentiation of female gametes. When the egg is mature, the orientation of the preformed axis is clearly visible; in animal eggs it corresponds to the animal-vegetal polarity, whereas in higher plant eggs it is reflected in the asymmetric localization of the vacuole. Thus, this fundamental process transpires within a single, relatively undifferentiated cell buried deep inside somatic tissue and not amenable to experimental manipulation.

Fucus zygotes offer several advantages. Large populations of fertilized eggs develop synchronously and are independent of other cells. During the first hours after fertilization, the orientation of the developmental axis is labile and can be experimentally manipulated by imposing external gradients on the zygotes (*1*). The most convenient polarizing vector is unilateral light; zygotes form an axis parallel to the light gradient. At 11 hours after fertilization, the axis becomes irreversibly fixed in space (*2*). In *Fucus* the axis determines the position of the rhizoid, which emerges from the spherical zygote at germination, 16 hours after fertilization. When unilateral light is applied, the rhizoid grows out from the dark hemisphere.

We recently developed a two-step procedure using cell wall–digesting enzymes for isolating homogenous populations of protoplasts from young *Fucus* zygotes (*3*). At the end of the 4.5-hour digestion period, no cell wall is visible by Calcofluor (a specific probe for β-linked glycosidic polymers) staining, electron microscopy, or birefringence in polarized light. When these protoplasts are placed in regeneration medium [RM; 60% artificial seawater (ASW) containing sucrose], synthesis of new cell wall is detected within 6 hours and is similar to wall assembly in normal zygotes (*4, 5*). Once the sucrose concentration in RM is lowered to less than 0.4*M*, the zygotes germinate, form normal rhizoids, and divide. Cells remain attached to the substratum throughout protoplast isolation and regeneration. We have used this procedure in conjunction with orienting pulses of unilateral light to investigate the role of the cell wall in establishing the polarity of the embryo.

We first investigated the requirement for cell wall in maintaining a fixed, polar axis. Zygotes developing in petri plates in ASW (*6*) were oriented by pulses of unilateral light that terminated at 11 hours, the end of the period of axis fixation (*2*). The cell wall was removed in two steps over 4.5 hours (11 to 15.5 hours), and the resulting protoplasts were allowed to regenerate a wall and germinate in uniform, nonorienting light (15.5 to 48 hours). At 48 hours the embryos were scored for the position of rhizoid outgrowth. When zygotes were polarized by unilateral light, rhizoids grew out parallel to one another on the shaded hemisphere (Fig. 1) (*7*). The percent polarization was calculated as the percentage of embryos bearing rhizoids on the shaded hemisphere. Control cells were treated as above except that no wall-digesting enzymes were added.

Short light pulses did not localize rhizoid outgrowth efficiently in any of the zygotes, probably because they did not receive sufficient energy for orientation. In these cases treated zygotes were not quite as well oriented as controls (the percent polarization was reduced by 10 and 17% in 3- and 5-hour light polarizations, respectively). However, polarization improved when the light pulse was longer; a 7.5-hour light

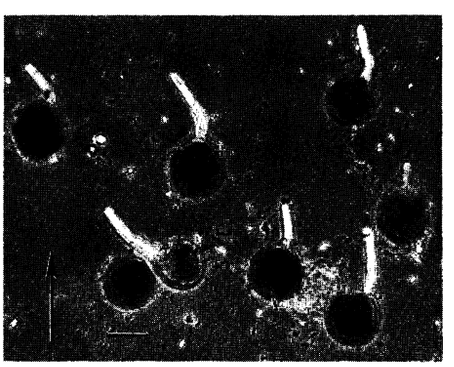

Fig. 1. Photopolarization of zygotes. Zygotes were given a pulse of unilateral light from 2 to 11 hours. Protoplasts were then obtained by treating zygotes for 1.5 hours in medium A [100 m*M* NaCl, 20 m*M* $MgCl_2$, 5 m*M* KCl, 250 μ*M* $CaCl_2$, 0.2% (w/v) bovine serum albumin (BSA), 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF), 10 m*M* tris, and 1*M* sorbitol, buffered to *p*H 7.8 with 2-(*N*-morpholino)ethanesulfonic acid (MES)] containing cellulase (CEL) (2 mg/ml) and alginate-lyase, H45-80 (1 unit/ml), followed by 3 hours in medium P [ASW containing 0.2% (w/v) BSA, 0.2 m*M* PMSF, 0.45*M* NaCl, 10 m*M* MES, buffered to *p*H 5.8 with tris] containing CEL (20 mg/ml) and alginate lyase (10 units of H45-80 and 0.1 unit of AG5-85 per milliliter). [See (*3*) for details of protocol.] Resultant protoplasts were placed in RM containing 0.8*M* sucrose for 12 hours, after which the sucrose concentration was lowered in steps of 0.2*M* every 4 hours. The regenerating protoplasts germinated on the dark side of the light pulse. Embryos were 4 days old when the photograph was taken. Arrow indicates direction of light vector. Scale bar, 50 μm.

Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331.

*To whom correspondence may be addressed.
†Present address: Centre d'Etudes Océanologiques et de Biologie Marine, CNRS (LP4601), 29211 Roscoff, France.
‡Present address: Central Research and Development, Experimental Station E402/2253, E. I. du Pont de Nemours & Company, Wilmington, DE 19898.