electrophoresis on an 8 percent polyacrylamide gel to resolve low molecular weight RNA's that were detected by autoradiography (Fig. 3B). VA-I RNA was synthesized at high levels in the adenovirus-infected cells. However, synthesis of the major endogenous cellular class III genes, 5*S* rRNA and tRNA genes, changed little with viral infection. We did not determine whether less abundant endogenous polIII genes were induced by viral IE proteins. Expression of a reiterated class III gene is increased in SV40-transformed mouse cell lines (*34*).

From these results we conclude that for class III genes as well as for class II genes, the expression of E1A proteins does not greatly affect the transcription of the major endogenous cellular genes, even though it has significant effects on the transcription of genes introduced by transfection. Berger and Folk (*35*) also observed that transcription of transfected VA-I and tRNA genes in 293 cells and in adenovirus-infected, Ara-C–treated HeLa cells was greater than that in uninfected HeLa cells.

Several investigators have reported that gene expression after transfection can be affected by factors such as altered stability of the transfected DNA in different cell lines (*36*) and the transfection technique used (*37*). To rule out such possible artifacts of transfection experiments, we analyzed the effect of viral IE proteins on class III gene transcription by another, independent approach. S100 extracts (*38*) were prepared from 143 cells and two cotransformed 143 cell lines, PR14 and PR15, which constitutively express the pseudorabies IE protein. When these extracts were assayed for in vitro transcription activity with exogenous class III gene templates, the PR14 and PR15 cell extracts were 10 to 20 times more active than extracts of the parental 143 cells (Fig. 4). This high in vitro polIII gene transcriptional activity of PR14 and PR15 cell extracts correlates well with the stimulation of class III gene transcription in transfection experiments in which PR14 and 143 cells are compared (Figs. 1 and 2). Other experiments showed that 293 cell extracts and extracts of adenovirus-infected, Ara-C–treated HeLa cells had significantly greater polIII transcriptional activity in vitro than extracts of uninfected HeLa cells (*39*). Thus, both transfection and in vitro transcription data demonstrate that viral immediate early proteins can activate class III gene transcription.

Analysis of VA-I and tRNA gene transcription in vitro has revealed that active templates are assembled into stable transcription complexes in which at least two protein factors are stably associated with the DNA template and do not dissociate and bind to a second polIII promoter added to an in vitro reaction (*40*). The E1A and IE proteins might activate class III genes by allowing more DNA molecules to be assembled into stable transcription complexes or by modifying a component of the stable transcription complexes, in such a way that the rate of transcription initiation from each complex is increased. Further analysis of extracts prepared from cells expressing E1A and IE proteins should distinguish between these and other possibilities.

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Synthesis of Chlorophyllide b from Protochlorophyllide in *Chlamydomonas reinhardtii* y-1

Abstract. *In cells of* Chlamydomonas reinhardtii *y-1 kept in the dark, 1,7-phenanthroline stimulated the conversion of protochlorophyllide to chlorophyllide b. A membrane fraction was obtained from degreened cells that was active in this conversion only when phenanthroline was present. Untreated cells excreted protochlorophyllide, which was used as substrate for this in vitro reaction. This system may provide a clue to how chlorophyllide b is synthesized in plant cells.*

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Although chlorophyll a (chl a) is found in all photosynthetic plant cells, its oxidized analog, chlorophyll b (chl b), is present only in the higher plants and green algae. Both forms are essential for proper assembly of the major light-harvesting chl-protein complex in chloroplast membranes (*1*). Chlorophyllide a (chlide a) (*2*) is synthesized from protochlorophyllide (pchlide) by a reduction of pyrrole ring D catalyzed by nicotinamide adenine dinucleotide phosphate (NADPH) pchlide oxidoreductase (Fig. 1) (*3*). In most higher plants, but only a few algal strains, this reaction requires light. Synthesis of chl b, however, remains an enigma; no enzymatic activity has been identified for this process, nor has the immediate precursor been determined. It has been presumed, but not

established, that chlide b is formed by oxidation of the methyl group on ring B of chlide a to an aldehyde (Fig. 1) (*4*). Conversely, it is possible that chlide b can be made from pchlide by an alternative pathway that does not involve chlide a. Our results reported here support the latter possibility.

The y-1 mutant strain of *Chlamydomonas reinhardtii*, which requires light for chl synthesis, was grown heterotrophically in the dark at 25°C. This reduces the level of chl below 1 nmol per 10^7 cells (*5*). When subsequently incubated at 38°C, these yellow cells, to our surprise, excreted pchlide into the medium at a rate of about 0.25 nmol per 10^7 cells per hour (Fig. 2A), 5 to 10 percent of the rate of chl synthesis in cells exposed to light (*5*, *6*). The absorbance spectrum for pchlide extracted from the medium had maxima at 434 and 622 nm (Fig. 2B). The fluorescence spectrum in diethyl ether had an excitation maximum at 436 nm and an emission maximum at 626 nm. These spectra are identical to those reported for pchlide from other plant tissues (*7*, *8*). Thin-layer chromatography of this material on silica gel yielded a single fluorescent spot (lane 2 in Fig. 2C). Further esterification with diazomethane (*9*) converted the material quantitatively to a more rapidly migrating methyl ester (lane 1 in Fig. 2C). These data identify the compound extracted from the medium as magnesium-2-vinyl-4-ethyl-pheoporphyrin a_5, or monovinyl pchlide (Fig. 1). Thus a convenient source of pchlide was available.

Yellow cells rapidly synthesized chlide b, but no chlide a, when treated in the dark at 38°C with 1,7-phenanthroline and similar analogs (*5*). Experiments were designed to examine the biosynthesis of chlide b in vivo and in vitro. Removal of acetate, the carbon source for biosynthetic reactions in the dark, from the medium (*10*) reduced de novo synthesis of porphyrins to about 10 percent of the rate in control cells (*5*). Thus for in vivo studies, cells were incubated in the dark in acetate-containing medium at 38°C for 1 to 2 hours, washed by centrifugation with medium lacking acetate, and then suspended in acetate-free medium. Cells obtained by this procedure contained an elevated pool of pchlide but did not synthesize a significant amount of additional porphyrins. With these conditions, we proceeded to determine whether phenanthroline directly stimulates conversion of pchlide to chlide b, as suggested by our previous results (*5*).

In cells treated with 10 m*M* 1,7-phenanthroline, a component with a fluorescence excitation maximum at 428 nm and an emission maximum at 657 nm in ethyl acetate (designated E428F657) appeared concomitant with a decrease in the amount of pchlide (emission maximum, 630 to 632 nm in ethyl acetate) (Fig. 3A). E428F657 is a demetalated chlide b–like pigment, probably monovinyl pheophorbide b (*5*). [The extraction procedure (*11*) caused loss of the Mg^{2+} ligand from the chlorin products but not from pchlide.] In companion cells exposed to light rather than phenanthroline, the product was primarily chl(ide) a (E410F670 in the demetalated form) (Fig. 3B).

Molecular oxygen was required for the conversion of pchlide to chlide b, as expected for oxidation of the methyl

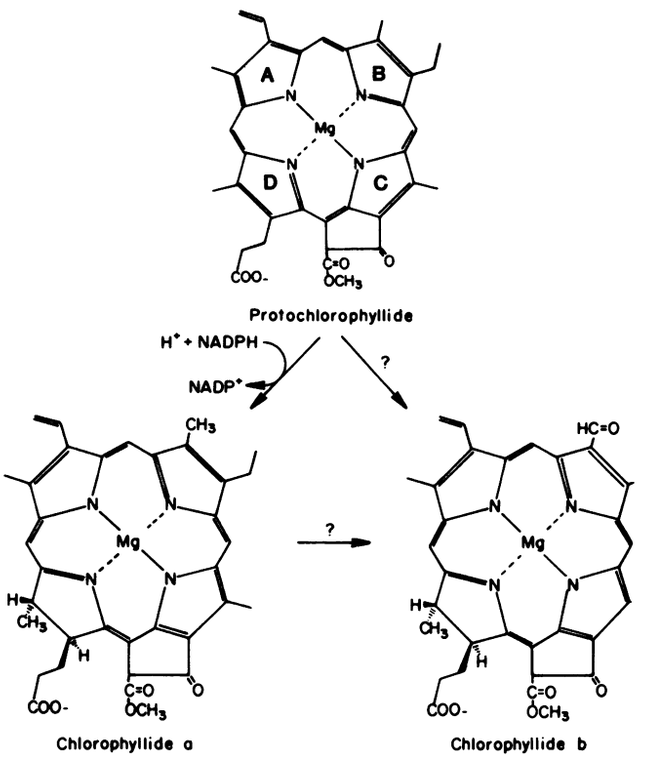

Fig. 1. Possible reactions in the synthesis of chlide b. Pchlide is converted to chlide a by the known photoreduction with NADPH of a double bond in pyrrole ring D, which contains the propionate side chain. Subsequent oxidation of the methyl group on ring B to an aldehyde group would generate chlide b. Alternatively, chlide a may not be an intermediate, and conversion of pchlide to chlide b may be initiated by an oxidation reaction.

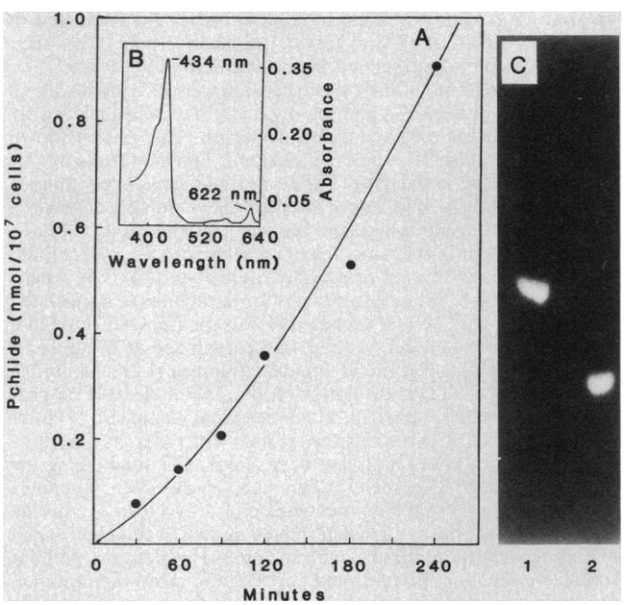

Fig. 2. (A) Accumulation of pchlide in the culture medium. Suspensions of yellow *C. reinhardtii* y-1 cells were incubated in the dark at 38°C (*5*). For each data point, cells were removed from 25-ml aliquots by centrifugation and the medium was extracted with 10 ml of diethyl ether. The concentration of pchlide was calculated from absorbance at 434 nm with extinction coefficient $\epsilon = 183\ mmol^{-1}$ (*7*). (B) Visible absorption spectrum of pchlide from the culture medium in diethyl ether. (C) Thin-layer chromatography of pchlide extracted from the medium on silica-gel type 60 plates (EM Laboratories), with toluene:ethyl acetate:ethanol:hexane (80:40:20:1 by volume) (*8*) as the mobile phase. Lane 1 shows migration (retardation factor R_F, 0.84) of pchlide esterified with diazomethane (*9*). Lane 2 shows the migration (R_F, 0.48) of untreated pchlide. The behavior of protoporphyrin IX (R_F, 0.19) and protoporphyrin IX dimethyl ester (R_F, 0.94), run on adjacent lanes as standards, shows that migration in this system was determined largely by the number of free carboxyl groups.

group. When cells were incubated with phenanthroline under N_2, production of chlide b was only 20 to 30 percent of that in cells treated under air. This amount still produced under N_2 may have been due to residual levels of O_2 remaining in the culture. However, synthesis of chlide a was not detected under these conditions. In contrast, when cells under N_2 were exposed to light instead of phenanthroline, pchlide was converted to chlide a without effect by the environment.

To further establish that phenanthroline promoted conversion of pchlide to chlide b, this process was examined in vitro. Yellow cells grown in the dark were exposed to light for 15 minutes to deplete the pool of endogenous pchlide and were then broken and fractionated into soluble and membrane fractions. These fractions were incubated separately with additional pchlide, which was collected from the culture medium as described in the legend to Fig. 2. As Fig. 4A shows, during a 30-minute incubation in the dark in the presence of phenanthroline, the membrane fraction converted most of the added pchlide to chlide b (E428F657 in the demetalated form). No activity was detected if the soluble fraction was substituted for the membrane fraction. Also, no reaction was detected when pchlide was incubated with all components except the membrane fraction, or when the membrane fraction was heated in boiling water for 1 minute before incubation with pchlide, or in the absence of phenanthroline. In reaction mixtures without phenanthroline but exposed to light, the product was chlide a. Omission of NADPH from the reaction mixtures reduced the production of chlide b.

Subcellular fractions also were prepared, with particular care taken to protect all samples from light in order to preserve the endogenous pchlide. In this case a yellow, fluorescent membrane fraction was collected from sucrose gradients that contained pchlide (about 4 nmol per milligram of protein) plus a small amount of residual chl (indicated by the peak of fluorescence near 665 nm in curve 1 in Fig. 4B). Incubation of this fraction alone for 30 minutes in the dark resulted in no spectral change, but in the presence of phenanthroline the pchlide was nearly completely converted to chlide b (curve 2 in Fig. 4B). Addition of hydroxylamine to a methanolic extract of a duplicate reaction mixture caused a shift in the emission spectrum from 657 to 666 nm, as expected for a formyl-containing product (*5, 12*).

Phenanthroline had no ability alone to convert pchlide to chlide b, but in the dark it promoted this process in combination with a membrane-bound cellular component. Synthesis of chlide b does not require light (*13*). If the reaction mixtures were exposed to even dim light during incubation, most of the chlorin produced was chlide a. This observation, plus the lack of synthesis of chlide a when cultures were treated in the dark with phenanthroline under N_2, indicated that chlide a was not an intermediate in the reaction. How phenanthroline brings about this effect is not known (*14*). However, these results suggest that a relatively large, aromatic compound facilitates chlide b synthesis.

Chlide b is not made in plant cells without prior synthesis of chlide a (*4, 13, 15*). Oelze-Karow *et al.* (*15*) summarized current thinking when they suggested that chlide a, when made at levels above a "threshold," acts as a homotropic effector for its own conversion to chlide b. However, an alternative possibility is that chlide a, when present in sufficient amounts, is a positive effector for the conversion of pchlide directly to chlide b. Phenanthroline may simply mimic chlide a in this process. Our data may provide a clue to how chlide b is synthe-

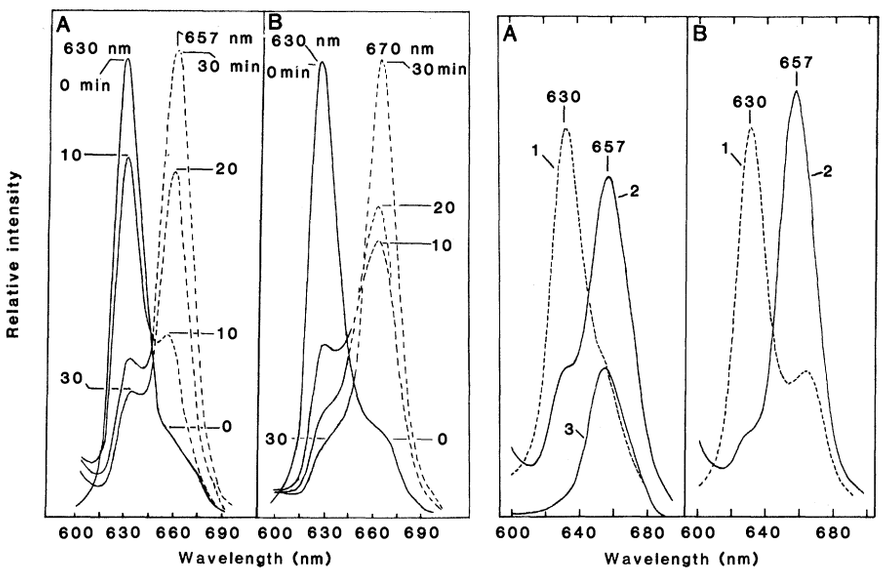

Fig. 3. (left) In vivo transformation of pchlide to chlide b. Yellow cells were incubated in acetate-containing medium (*5, 10*) in the dark at 38°C to elevate the pchlide pool. Cells then were washed twice with acetate-free medium, suspended in this medium (1×10^7 cells per milliliter), and incubated at 38°C. Aliquots (25 ml) of the cell suspension were extracted with 10 ml of ethyl acetate : acetic acid (3:1 by volume) (*11*) and the organic phase was analyzed by fluorescence spectroscopy (*5*), with 434 nm as the excitation wavelength. The spectra shown are uncorrected for response of the photomultiplier versus wavelength. For the results in (A), the cells were treated with 1,7-phenanthroline in the dark. The decrease in pchlide (continuous lines) occurred concomitantly with an increase in chlide b (dashed lines). In (B), cells were exposed to light rather than treated with phenanthroline, and the major product was chl(ide) a (dashed lines). In untreated suspensions kept in the dark, the amount of pchlide or of chl(ide) did not change. Fig. 4. (right) In vitro conversion of pchlide to chlide b. (A) Yellow cells were incubated in the dark at 38°C, exposed to incandescent light for 15 minutes to deplete the cellular pool of pchlide, washed twice in cold 50 m*M* Hepes (*p*H 7.2), and passed through a French pressure cell at 4×10^7 Pa. The broken cell sample was centrifuged at 2000*g* for 3 minutes to remove whole cells and then at 100,000*g* for 30 minutes. Samples of the membrane fraction (0.5 mg of protein) were incubated at 38°C in the dark with 10 μ*M* pchlide, 0.15 percent sodium cholate, 0.5 m*M* NADPH, and 10 m*M* 1,7-phenanthroline in a final volume of 100 μl of 50 m*M* Hepes (*p*H 7.2). Pchlide was obtained by extracting the medium with ether, as described in the legend to Fig. 2B, drying the extract under a stream of nitrogen, and solubilizing the residue in 50 m*M* Hepes (*p*H 7.2) containing 0.25 percent sodium cholate (*3*). After 30 minutes the reaction mixtures were extracted with ethyl acetate : acetic acid (4:1 by volume), and the fluorescence emission spectra of the organic phases were determined as described in the legend to Fig. 3. Curve 1 represents a control reaction mixture with membrane protein but without phenanthroline; curve 2, the complete reaction mixture; and curve 3, the membrane fraction alone. (B) Cells were protected from light throughout the procedure to preserve endogenous pchlide. The 2000*g* supernatant was placed onto a discontinuous gradient of 15 ml of 1.5*M* sucrose and 15 ml of 1.0*M* sucrose in 50 m*M* Hepes (*p*H 7.2) and spun at 78,000*g* for 3 hours. Samples of membrane material (1 mg of protein and 4 nmol of pchlide) were collected from the interface, incubated in 0.5 ml of 50 m*M* Hepes (*p*H 7.2), and assayed as before. Curve 1, without phenanthroline and curve 2, with 10 m*M* 1,7-phenanthroline present during incubation.

sized in plant tissues and how the ratio of chl a to chl b is regulated during development of the chloroplast.

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Inhibition of Lymphocyte Proliferation by a Synthetic Peptide Homologous to Retroviral Envelope Proteins

Abstract. *The retroviral transmembrane envelope protein p15E is immunosuppressive in that it inhibits immune responses of lymphocytes, monocytes, and macrophages. A region of p15E has been conserved among murine and feline retroviruses; a homologous region is also found in the transmembrane envelope proteins of the human retroviruses HTLV-I and HTLV-II and in a putative envelope protein encoded by an endogenous C-type human retroviral DNA. A peptide (CKS-17) was synthesized to correspond to this region of homology and was examined for its effects on lymphocyte proliferation. CKS-17 inhibited the proliferation of an interleukin-2–dependent murine cytotoxic T-cell line as well as alloantigen-stimulated proliferation of murine and human lymphocytes. Four other peptides, representing different regions of virus proteins, were inactive. These results suggest that the immunosuppressive portion of retroviral transmembrane envelope proteins may reside, at least in part, in a conserved sequence represented by the CKS-17 peptide.*

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Immunosuppression frequently accompanies retroviral infections and often precedes the development of neoplasms in animals infected by oncogenic retroviruses (*1*). Although the mechanisms are not yet known, there is increasing evidence that the envelope protein p15E participates in the pathogenesis of retroviral-induced immunosuppression (*2*). P15E is a hydrophobic transmembrane protein approximately 19,000 daltons in size that is cleaved from an envelope precursor polypeptide of approximately 80,000 to 90,000 daltons (*3*). Lymphocyte blastogenic responses to mitogens and alloantigens are inhibited by isolated p15E of feline leukemia virus (FeLV), and the transformation of human lymphocytes by concanavalin A is blocked by FeLV p15E (*4, 5*). Inhibition of proliferation of a murine cytotoxic T-lymphocyte (CTL) cell line by a p15E-like protein from FeLV has also been reported (*6*). Furthermore, murine leukemia virus (MuLV) p15E inhibits macrophage accumulation at inflammatory foci in mice (*7*). Proteins with similar anti-inflammatory properties that are antigenically and physicochemically related to p15E have been identified in virus-free murine tumor cell lines and primary murine tumors (*8*). Human cancerous effusions contain p15E-related proteins that inhibit the responses of human monocytes to chemotactic stimuli (*9*), and p15E-related proteins have been identified in human malignant cells and in the plasma of leukemic patients (*10, 11*). It has therefore been postulated that certain tumor cells, not exogenously infected with retroviruses, may produce immunosuppressive products related to p15E and thereby evade immune surveillance (*2*).

Human T-cell leukemia/lymphoma virus (HTLV) is the designation for a family of lymphotrophic, exogenous retroviruses (*12*). Immunosuppression often accompanies HTLV-associated lymphocyte malignancies, and coculture of normal human lymphocytes with ultraviolet-irradiated HTLV-I inhibits their blastogenic responses to mitogens (*13*). The transmembrane envelope protein of HTLV-I and HTLV-II, gp21E, shares significant amino acid sequence homolo-

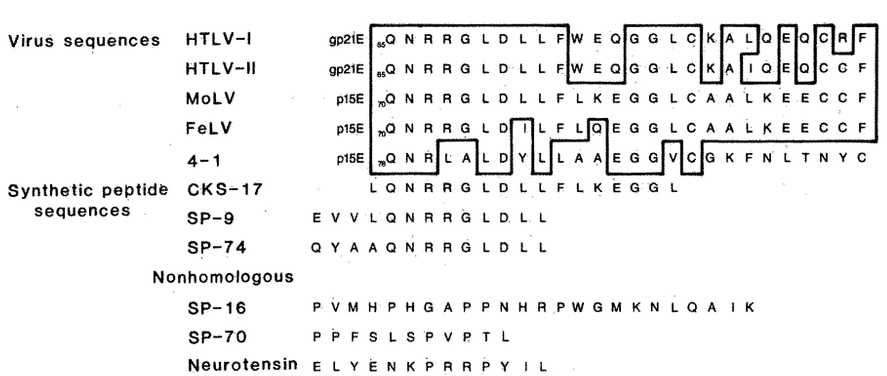

Fig. 1. Amino acid sequences (*19*) for the conserved region of retrovirus transmembrane envelope proteins gp21E and p15E and partially homologous or nonhomologous synthetic peptides. MoLV, Moloney murine leukemia virus. The number at the lower left of each sequence represents the residue at which the sequence begins.