I and Sph I sites in the pGEM3 polylinker and ligated it to a 5- to 7.5-fold molar excess of each of duplexes L and R. Excess oligomers were removed from the 5.6-kbp DNA by precipitation in the presence of polyethylene glycol. The plasmid form of the 5.6-kbp cyclization substrate, incorporating duplexes L and R, was designated pCC56cos. The plasmid pCC86 (8.8 kbp) consists of a 5.9-kbp Sal I–Eco RI fragment from the λ genome ligated to a 2.8-kbp Sal I–Eco RI fragment from pGEM3. The 8.6-kbp cyclization substrate was constructed by ligation of duplexes L and R onto an 8.5-kbp Sph I–Xho I fragment of pCC86; subsequent purification of the 8.6-kbp substrate was identical to that of its 5.6-kbp counterpart described above.

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28. Cyclization of radiolabeled DNA was carried out by heating at 70°C for 5 to 7 min to unpair any preexisting cohered single-stranded ends and then annealing for 6 to 8 hours at 34°C. Unlabeled linear and *Saccharomyces cerevisiae* DNA topoisomerase II–knotted (*14*) pCC56cos DNA were added to the labeled samples as carriers before electrophoretic analysis in agarose gel at 4°C, as described in (*14*). Gel slabs were dried and analyzed in a Phosphor-Imager (Molecular Dynamics); the radioactivity of the various bands was quantitated with MD ImageQuant Software version 3.15.
29. T4 DNA topoisomerase was used to generate a series of supercoiled knots from negatively supercoiled pCC56cos as described (*25, 26*); these were then nicked by Hind III in the presence of ethidium bromide (*27*), and the nicked trefoil was gel-purified. The trefoil was radiolabeled by nick translation and ligated by *Escherichia coli* DNA ligase. Electrophoresis was done at room temperature in 0.9% agarose gels in the presence of 90 mM tris base, 90 mM boric acid, and 1.8 mM Na_2EDTA.

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Negative Regulation of G1 in Mammalian Cells: Inhibition of Cyclin E–Dependent Kinase by TGF-β

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Transforming growth factor–β (TGF-β) is a naturally occurring growth inhibitory polypeptide that arrests the cell cycle in middle to late G1 phase. Cells treated with TGF-β contained normal amounts of cyclin E and cyclin-dependent protein kinase 2 (Cdk2) but failed to stably assemble cyclin E–Cdk2 complexes or accumulate cyclin E–associated kinase activity. Moreover, G1 phase extracts from TGF-β–treated cells did not support activation of endogenous cyclin-dependent protein kinases by exogenous cyclins. These effects of TGF-β, which correlated with the inhibition of retinoblastoma protein phosphorylation, suggest that mammalian G1 cyclin-dependent kinases, like their counterparts in yeast, are targets for negative regulators of the cell cycle.

Progression through the cell cycle is regulated by cyclin-dependent protein kinases (CDKs) (*1*). The CDKs are necessary for the start of the S phase (*2*) and mitosis (*3*). The mitotic role of the CDKs is thought to be executed by the prototypic CDK, Cdc2 (*3, 4*). Cdc2 is positively regulated by cyclin B (*5*), which is synthesized during the S and G2 phases. The binding of cyclin B to Cdc2 induces phosphorylation of the complex, which leads to its activation at the G2 to M transition (*6, 7*). In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, this kinase is also necessary for starting the S phase (*8*). In higher eukaryotes, however, the CDKs have evolved into a small gene family (*9*), and at least one additional family member, Cdk2, also functions during the S phase (*10, 11*). Cdk2 kinase activity is first evident during the middle of G1 (*12, 13*), in which it may phosphorylate the retinoblastoma gene product (Rb) (*14–16*). Cdk2, like Cdc2, is regulated by its association with cyclins. It assembles in a complex with cyclin E in the middle of G1 (*13*); this is followed at the start of the S phase by formation of a complex with cyclin A (*11, 12, 17*). Cyclin E–Cdk2 may function in controlling progression through G1 (*18*), and cyclin A–Cdk2 may function in controlling the start of DNA synthesis (*19*).

Transforming growth factor–β inhibits cell proliferation by delaying or arresting progression through the late portion of G1 (*20, 21*). In Mv1Lu lung epithelial cells, TGF-β prevents Rb phosphorylation during G1 (*21*), retaining Rb in a hypophosphorylated state that may suppress progression into the S phase (*15, 16, 22*). Because the activity of cyclin-dependent kinases seems critical for the G1 to S transition (*13, 18, 19*), negative regulators of this transition might operate by affecting the activity of these kinases. We investigated whether a known G1 kinase, the cyclin E–Cdk2 complex, might be inhibited by TGF-β.

Proliferating Mv1Lu cells were arrested by growth to high density in serum-containing medium. We then released these contact-inhibited cells from quiescence by sparsely seeding them in this medium. As reported (*21*), the transit through the middle of G1, 6 hours after the release from contact inhibition, involved Rb phosphorylation and was blocked by TGF-β added at 6 hours (Fig. 1A). Protein immunoblots with cyclin E antibodies and Cdk2 antibodies showed that these proteins were present in contact-inhibited cells (Fig. 1B). As cells progressed through G1, a faster migrating form of Cdk2 appeared (Fig. 1B) that corresponds to the phosphorylated, catalytically active state of this protein (*23*). To follow the kinetics of Cdk2 association with cyclin E, we immunoprecipitated cell extracts with cyclin E antibodies and analyzed the immune complexes by immunoblot using Cdk2 antibodies (*13*) (Fig. 1C). A small amount of slow-migrating Cdk2 form was present in cyclin E immune complexes 3 hours after the start of G1 and thereafter (Fig. 1C, inset). The amount of fast Cdk2 form associated with cyclin E increased 6 hours after the start of G1 and became the predominant cyclin E–associated Cdk2 form 9 hours after the start of G1 and later (Fig. 1C). The increase in cyclin E–associated fast Cdk2 form was correlated closely with an increase in cyclin E–associated histone H1 kinase activity (Fig. 1D) over a basal background level and preceded by several hours the appearance of kinase activity precipitable with Cdc2 antibodies. Thus, the natural progression of Mv1Lu cells from quiescence into the S phase

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involves acquiring the capacity to assemble active cyclin E–Cdk2 complexes from the steady-state levels of their components.

The addition of TGF-β did not decrease the amount of cyclin E or Cdk2 (Fig. 1B). However, cells that received TGF-β at the start of G1 or 6 hours after cell entry into G1 did not accumulate the faster migrating form of Cdk2 (Fig. 1B), did not assemble cyclin E–Cdk2 complexes (Fig. 1C), and did not contain cyclin E–associated histone kinase activity (Fig. 1D). We could not detect any Cdc2 in cyclin E immune complexes from cells in the middle of the G1 phase by protein immunoblot with Cdc2 antibody (*13*). Because cyclin E does not have any known partner during G1 other than Cdk2 (*13*), the cyclin E–associated H1 kinase activity that is down-regulated by TGF-β during G1 corresponds largely if not exclusively to the activated cyclin E–Cdk2 complex. Thus, TGF-β may prevent Cdk2 activation in Mv1Lu cells by interfering with the stable association of preexisting cyclin E with Cdk2 and the activation of this complex.

To test the ability of TGF-β to inhibit cyclin E–Cdk2 activation, we added exogenous cyclin E to cell extracts, and histone kinase activity was assayed in immunoprecipitates of Cdk2 antibodies from these incubation mixtures (Fig. 2A). Addition of cyclin E generated a greater amount of Cdk2-associated histone kinase activity in extracts from control cells in late G1 (18 hours after release from contact inhibition) than in extracts from control cells in mid-G1 (6 hours after release from contact inhibition), which is correlated with the activation of the endogenous cyclin E–Cdk2 kinase complex. The specificity of the cyclin E and Cdk2 interaction was confirmed by the inability of cyclin E to activate endogenous Cdc2 in late G1 extracts even though addition of cyclin A to these extracts readily activated endogenous Cdc2 and Cdk2 (Fig. 2A).

We compared the activity of late G1 extracts to that of extracts from cells at an equivalent time point that had been exposed to TGF-β from the middle of G1 or earlier. Addition of cyclin E to extracts from TGF-β–treated cells did not result in activation of the endogenous Cdk2 (Fig. 2A). Activation of endogenous Cdk2 or Cdc2 by exogenous cyclin A was also blocked in extracts from TGF-β–treated cells. The failure to generate active kinase in these extracts was not because of the absence of Cdk2 or degradation of added cyclin E, as determined by immunoblot (*24*). In fact, immunoprecipitation with antibodies to cyclin E followed by immunoblot with antibodies to Cdk2 showed that exogenous cyclin E formed a complex

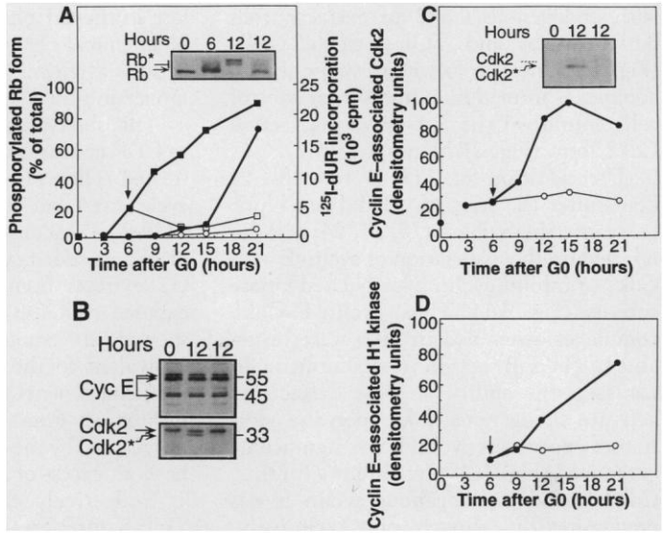

Fig. 1. Effect of TGF-β1 on cyclin E–associated Cdk2 and protein kinase activity during the G1 phase. Mv1Lu cells were released from G0 by being placed in growth medium (minimal essential medium with 10% fetal bovine serum) for the indicated times (closed symbols). Some cultures received 100 pM TGF-β1 6 hours after release from G0 (arrows) or when otherwise indicated, and incubations continued for the indicated times (open symbols). (**A**) Kinetics of Rb phosphorylation and S-phase entry. Rb protein was analyzed by immunoblot (*21*) (inset) of cell extracts from the indicated time points with Rb antibody (PharMingen G3-245, San Diego, California) and developed with the ECL detection system (Amersham). In the inset, time after G0 is indicated at the top; TGF-β was added to the last lane 6 hours later. The relative proportion of hyperphosphorylated (Rb*) Rb form (squares) was determined by densitometry of the films. We assessed entry into the S phase by measuring the level of [^{125}I]-labeled deoxyuridine (^{125}I-dUR) incorporated into DNA (*21*) during the last hour of the incubations (circles). (**B**) Cyclin E (Cyc E) and Cdk2 amounts in Mv1Lu cells during G1 determined by immunoblot (*29*). Hours are as in the inset in (A); TGF-β was added to the last lane at time (t) = 0. Cdk2*, fast Cdk2 form. Molecular size markers are shown in kilodaltons. (**C**) Cyclin E–associated Cdk2 was determined by immunoprecipitation of cell extracts with affinity-purified rabbit antibodies to human cyclin E coupled to CNBr-activated sepharose followed by protein immunoblot of these samples with antibodies to human Cdk2. The amount of the fast Cdk2 form (inset) was determined by densitometry of the films and is presented in arbitrary units. In the inset, hours are as in (A); TGF-β was added to the last lane at t = 0. (**D**) Cyclin E–associated histone H1 kinase activity was determined by precipitation of cell extracts with rabbit antibodies to human cyclin E and kinase reactions with these samples in the presence of [γ-^{32}P]adenosine triphosphate and histone H1 (*30*). After stopping the reactions, we electrophoresed the samples on SDS-polyacrylamide gels and quantitated the radiolabeled histone H1 band with a PhosphorImager and ImageQuant software (Molecular Dynamics).

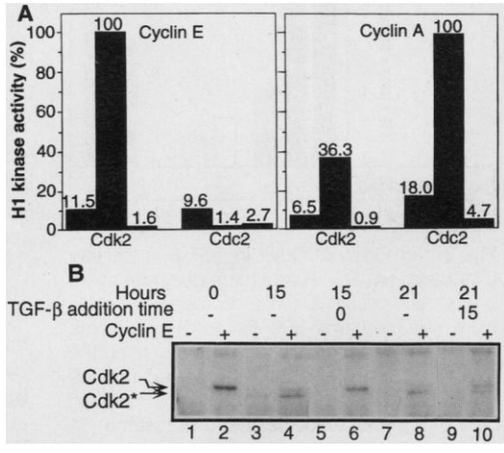

Fig. 2. G1 phase extracts from TGF-β–treated cells did not support activation of endogenous CDKs by exogenous cyclins. (**A**) Cells were released from G0 for 6 hours (left bars in each group of three bars) or 18 hours (middle bars) with no additions or for 18 hours with TGF-β1 addition at 6 hours (right bars). Cell extracts were prepared from these cells by hypotonic lysis (*30*) and incubated for 30 min with either recombinant cyclin E or recombinant cyclin A obtained from baculovirus-infected cell systems (*31*). The amount of cyclin added to these extracts was adjusted to correspond to the amount of cyclin present in a similar amount of MANCA cells in the S phase (*13*). After the incubations, the reaction mixtures were adjusted to immunoprecipitation buffer conditions [0.5% NP-40, 250 mM NaCl, 20 mM tris (pH 7.4), 0.3 mM vanadate, and 50 mM NaF] and divided for immunoprecipitation with either affinity-purified Cdc2 COOH-terminus antibodies or Cdk2 COOH-terminus antisera (*13*). Immunoprecipitates were used in histone H1 kinase assays (*30*). After stopping the reactions, we electrophoresed the samples on SDS-polyacrylamide gels and quantitated the radiolabeled histone H1 band with a PhosphorImager. We corrected all values by subtracting the values obtained in incubations without added cyclin and plotted them as the percent of the maximal activity value. (**B**) After incubating cell extracts with exogenous cyclin E as described above, we subjected aliquots of the incubation mixtures to immunoprecipitation with cyclin E antibody followed by immunoblot with Cdk2 antibody. Designations are as in the insets in Fig. 1.

with endogenous Cdk2 in extracts from both control and TGF-β–treated cells (Fig. 2B, lanes 1 to 6). However, only the complexes formed in extracts from control cells contained the fast-migrating, active Cdk2 form (Fig. 2B, lanes 1 to 6).

The addition of TGF-β to cells 15 hours after the start of G1 did not block entry into the S phase (*20, 21, 24*) and did not reverse the association of cyclin E with Cdk2 or inhibit cyclin E–associated kinase activity (Fig. 3A). Thus, cyclin E–Cdk2 complexes assembled in vivo were resistant to TGF-β action once formed. In contrast, the ability of these extracts to activate endogenous Cdk2 upon the addition of exogenous cyclin E was significantly reduced (Fig. 3B). Consistent with this, Cdk2 bound to exogenous cyclin E was predominantly, though not exclusively, the slower mobility form (Fig. 2B, lanes 7 to 10). Therefore, cells exposed to TGF-β

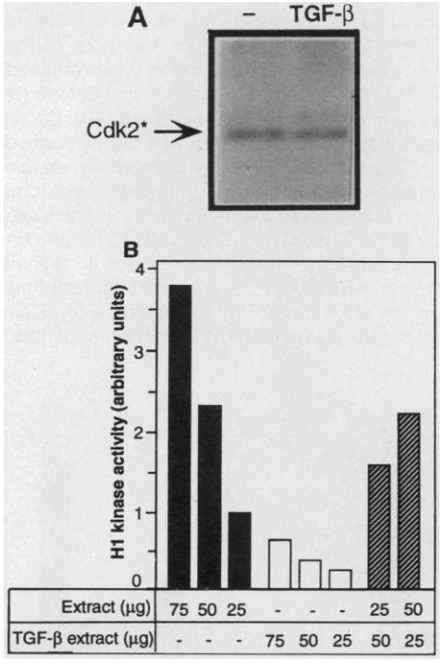

Fig. 3. Sensitivity of Cdk2 to TGF-β in the late G1 phase. (**A**) TGF-β treatment does not affect preformed cyclin E–Cdk2 complexes. Cells were released from G0 for 21 hours; some cultures received TGF-β1 15 hours after the release (TGF-β), and others received nothing (–). Immunoprecipitation of cyclin E followed by Cdk2 immunoblot assays and histone H1 phosphorylation assays were performed in extracts from these cells as described in Fig. 1. H1 kinase activity in arbitrary units was 1.0 and 1.1 for the first and second lanes, respectively. Cdk2* is as in Fig. 1. (**B**) TGF-β treatment inhibits the activation of Cdk2 by exogenous cyclin E. The indicated amounts (in micrograms of protein) of extracts from these two sets of cells were assayed separately or mixed for Cdk2-associated histone H1 kinase activity. Assays were done as indicated (Fig. 2A).

late in the G1 phase continued to exhibit biochemical changes in cyclin-CDK interactions when cell cycle progression was no longer inhibited by TGF-β.

This observation suggested that the block to CDK activation in extracts from TGF-β–treated cells was not a consequence of cell cycle arrest but perhaps a direct effect of TGF-β. To address the mechanism of this block, we added cyclin E to mixtures of late G1 extracts from control cells and cells exposed to TGF-β at 15 hours. The mixtures showed an amount of Cdk2 activation equivalent to the sum of the activities of their components (Fig. 3B). Thus, although the extract from TGF-β–treated cells was not rescued by the control extract, it did not have an excess of a Cdk2 inhibitor.

Collectively, these results indicate that TGF-β interferes with cyclin association with the CDKs in G1 and activation of these complexes. In vivo, TGF-β prevents the stable assembly of cyclin E–Cdk2 complexes, and this results in the absence of the cyclin E–associated kinase activity that normally accumulates in G1. In extracts from TGF-β–treated cells, exogenously added cyclin E can assemble into complexes with endogenous Cdk2, but the Cdk2 is not fully phosphorylated and these complexes do not have kinase activity. We interpret these results to suggest that the primary effect of TGF-β is to inhibit phosphorylation of Cdk2 on an essential threonine residue (*6, 7, 23*) and thereby prevent activation of cyclin-CDK complexes. The failure of cyclin E to form a stable complex with Cdk2 in TGF-β–treated cells is probably a result of a lower affinity of cyclin E for Cdk2 when Cdk2 is not phosphorylated, as reported for cyclin B–Cdc2 complexes (*7, 25*).

Although the lack of Cdk2 phosphorylation in TGF-β–treated cells might be because of the loss of a kinase that phosphorylates Cdk2, this hypothesis would not explain the inability of control extracts to rescue extracts from TGF-β–treated cells in mixing experiments. Thus, TGF-β treatment may render Cdk2 intrinsically incompetent to undergo activation by cyclin E because of posttranslational modification or a block of Cdk2 imposed by a third protein; Rb-related proteins can bind certain cyclins and CDKs, thereby establishing potential regulatory feedback loops (*26*).

The inhibition of cyclin E–Cdk2 by TGF-β established here is the first example of a mammalian G1 kinase being negatively regulated by a physiological agent. In vitro TGF-β inhibitory effects extend to the ability of cyclin A to activate both Cdk2 and Cdc2. TGF-β may cause a general inhibition of cyclin-CDK interactions during G1 that would lead to inhibition of Rb phosphorylation and delay or arrest of G1 progression, which may favor cell differentiation (*27*). The analogy between this mechanism and the ability of yeast mating pheromones to arrest the cell cycle in late G1 by down-regulating CLN-Cdc28 activity (*28*) indicates that G1 cyclin-dependent kinases may constitute general targets for negative regulators of the eukaryotic cell cycle.

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29. Cells (2×10^6) were lysed in SDS–radioimmunoprecipitation assay buffer as previously described by Koff *et al.* (*13*) and electrophoresed on 12% polyacrylamide gels. Proteins in the gels were electroblotted onto polyvinyl difluoride membranes and blocked for 2 hours with 5% milk in TNT [25 mM tris (pH 7.5), 150 mM NaCl, 0.05% Tween 20]. Membranes were probed overnight with cyclin E antiserum diluted 1:1000 in TNT containing 1% Tween 20. Bound antibody was detected with enhanced chemiluminescence (ECL) (Amersham) according to the manufacturer's directions. The specificity of the cyclin E bands was verified by their absence in identical blots probed with cyclin E antiserum that had been preincubated with glutathione-S-transferase–cyclin E agarose (*13*) for 4 hours at 4°C in TNT. To determine the amount of Cdk2, we lysed cells in hypotonic buffer and sonicated them for 1 min. Cell debris was removed by centrifugation at 100,000*g*, and samples of the supernatants (10 μg of protein) were subjected to immunoblot as described above for cyclin E except that Cdk2 antiserum (*13*) was used as the primary antibody.
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32. The first two authors contributed equally to this work. A.K. is the recipient of a postdoctoral fellowship from NIH. M.O. is a visiting scientist sponsored by Sankyo Co., Ltd., and J.M.R. is a Lucille P. Markey scholar in the biomedical sciences. J.M. is a Howard Hughes Medical Institute investigator. Support for this work was provided by grants from the Lucille P. Markey Charitable Trust and from NIH (J.M.R. and A.K.) and by the Howard Hughes Medical Institute (J.M.).

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Reversible Binding of Nitric Oxide by a Salivary Heme Protein from a Bloodsucking Insect

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The bloodsucking bug *Rhodnius prolixus* has a salivary vasodilator, previously characterized as a nitrovasodilator, with salivary smooth muscle–relaxing and antiplatelet activity. *Rhodnius* salivary glands are bright red owing to the abundance of heme proteins. Electron paramagnetic resonance and optical spectroscopic experiments indicated that the salivary vasodilator is a nitrosylheme protein with an Fe(III) heme that binds nitric oxide (NO) reversibly. Dilution of the protein in neutral pH promoted NO release. This protein thus appears to be the NO carrier that helps *R. prolixus* to feed on blood.

The bloodfeeding bug *R. prolixus* has a salivary vasodilator with the general properties of NO (*1*). Because NO has a half-life on the order of seconds in the presence of aerated solutions (*2*), the question arises as to the identity of the parent molecule that ultimately releases the vasodilator NO. On account of an abundance of heme proteins that give *Rhodnius* salivary glands a deep cherry color (*3*), we tested the hypothesis that the *Rhodnius* vasodilator is a nitrosylheme protein that not only binds but also can readily release NO.

Optical spectroscopy of native *Rhodnius* salivary gland homogenates at pH 5.0 and 7.35 indicated typical heme spectra, with a Soret band maximum at 422 nm. After dilution, this maximum shifted to 406 nm only at the more alkaline pH, indicating a change in ligation of the heme in the alkaline pH range (Fig. 1). This dilution shift was rapid, and the resulting spectrum was stable for at least 30 min at room temperature.

We reasoned that if the heme protein was in equilibrium with free NO gas, then addition of a neutral gas to this solution should displace the NO and change the spectra. Indeed, when the native, concentrated homogenate at pH 7.4 was exposed to an argon atmosphere (*4*), the Soret maximum shifted from 422 to 406 nm, indicating volatile ligand displacement by argon. When this unliganded heme protein was subsequently exposed to NO for 1 min, the Soret maximum shifted back to 422 nm, identical to the original spectrum of the native protein (Fig. 2). When reexposed to argon, the Soret maximum of the reconstituted nitrosylheme protein again shifted to 406 nm, indicating loss of the NO group once more (Fig. 2). The same shift of the Soret maximum was also produced by dilution of the reconstituted nitrosylheme protein at alkaline pH. However, this shift did not occur at pH 5.0, a result identical to that obtained for the native homogenate shown in Fig. 1. These results support the hypothesis that the native homogenate contains a nitrosylheme protein with relatively low affinity for NO.

The Soret band shifts shown in Figs. 1 and 2 are similar to those observed for Fe(III) heme proteins, for example, oxidized myoglobin (metMb; 409.5 nm) to metMbNO (420.5 nm), and are opposite to the shifts observed for Fe(II) heme proteins, for example Mb (435.0 nm) to MbNO (421.5 nm) (*5*). The Soret band shifts are not consistent with either Fe(II) or Fe(III) spectral shifts of cytochrome P450 (*6*).

To investigate further the pH effect on the spectral changes after dilution of the homogenate, we used a simple model of two heme protein fractions that either absorb maximally at 406 nm (and with spectrum

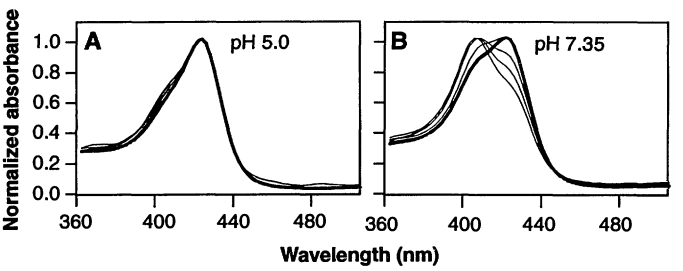

Fig. 1. Normalized spectra of *R. prolixus* salivary gland homogenates serially diluted at pH 5.0 (**A**) or 7.35 (**B**). The most concentrated sample is shown with a thicker line. Ten pairs of glands [in 20 μl of 10 mM sodium phosphate buffer and 150 mM NaCl (pH 7.2)–phosphate-buffered saline (PBS)] were added to 0.5 ml of buffer (0.1 M citrate buffer (pH 5.0) or 0.1 M Hepes buffer (pH 7.35), and spectra at 2-nm intervals were recorded in a diode array spectrophotometer (Hewlett-Packard 8452A) from 360 to 500 nm. The samples were then serially diluted twofold in the same buffer to obtain the various spectra shown. Absorbance of the concentrated samples was 1.2 absorbance units for a cuvette of path length 1 cm. The same results were obtained when acetate was used instead of citrate and phosphate instead of Hepes as buffer.

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