These findings represent direct evidence that *Ae. albopictus* may be an arbovirus vector in the United States. Recovery of the virus from mosquitoes does not establish this species as a vector in the United States; however, laboratory studies have demonstrated that *Ae. albopictus* can transmit Potosi virus (*24*). At the same time, experiments demonstrating that the virus is not vertically transmitted by *Ae. albopictus* provide evidence that this species is unlikely to serve as a reservoir and overwintering host for the virus. Vertebrates that may serve as hosts for Potosi virus, or other mosquito species that may serve as natural vectors, are currently unknown.

The important question of whether the virus will infect humans remains to be addressed. Preliminary testing of field workers exposed during mosquito collection showed that 1 of 16 tested had neutralizing antibody to the new virus. It is unclear whether this person was infected during field studies in Potosi or was previously infected with a cross-reactive virus.

Although a major concern has been the involvement of *Ae. albopictus* as a vector of LAC virus to humans, the possibility remains that other viruses, which have heretofore not infected humans to any appreciable degree because of the nonhuman feeding habits of their arthropod vectors, may be transmitted to humans by *Ae. albopictus* as a result of the aggressive feeding of this species on humans as well as on other mammalian hosts.

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25. We thank J. Cully, S. Hanson, W. Hawley, J. P. Mutebbe, C. Pumpuni, and T. Streit, who participated in the field collection of mosquitoes from Potosi, Missouri, during this study.

31 July 1990; accepted 5 November 1990

Regulation of Progesterone Receptor-Mediated Transcription by Phosphorylation

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The progesterone receptor (PR) in the chicken oviduct is a phosphoprotein that regulates gene transcription in the presence of progesterone. Treatment with progesterone in vivo stimulates phosphorylation of the progesterone receptor. With transient transfection assays, the present work has tested whether phosphorylation participates in the regulation of PR-mediated transcription. Treatment with 8-bromo-cyclic adenosine monophosphate (8-Br cAMP), a stimulator of cAMP-dependent protein kinase [protein kinase A (PKA)], mimicked progesterone-dependent, receptor-mediated transcription in the absence of progesterone. Inhibition of PKA blocked hormone action. Treatment with okadaic acid, an inhibitor of protein phosphatases 1 and 2A, stimulated transcription in a manner similar to that of progesterone. These observations suggest that phosphorylation of the PR or other proteins in the transcription complex can modulate PR-mediated transcription in vivo.

THE STEROID HORMONE RECEPTORS are members of a superfamily of transcriptional regulatory factors (*1*). These receptors are phosphoproteins (*2–4*) that transduce the signal carried by their cognate hormone, resulting in regulation of gene expression. The general organization of the PRs is shown in Fig. 1. Receptors isolated from chicks treated with progesterone show increased phosphorylation, decreased mobility on SDS-polyacrylamide gels, decreased interaction with heat shock protein 90 (hsp90) in vitro, and increased DNA-binding activity (*2, 3*). The PR has three hormonally regulated sites, two in the NH_2-terminal domain and one in the COOH-terminal transactivation domain (*5*).

The present studies assess the function of phosphorylation in regulation of PR-mediated transcription in vivo. Because progesterone stimulates phosphorylation of the PR, we tested whether stimulation of protein kinases could regulate progesterone-dependent, receptor-mediated transcription of target genes. Monkey kidney CV1 cells, which do not express an endogenous PR, were cotransfected with a PR_A expression plasmid and a reporter gene that contained the progesterone response element (PRE) upstream of the chloramphenicol acetyltransferase (*CAT*) gene (*6*). Treatment of cells with progesterone resulted in a marked induction of PR_A-mediated transcription (Fig. 2A). Treatment with progesterone and 8-bromo-cyclic adenosine monophosphate (8-Br cAMP), an activator of protein kinase A (PKA), resulted in twice as much transcription. In the absence of progesterone, 8-Br cAMP caused an induction similar to that elicited by progesterone (about 20-fold).

Since others have shown that 8-Br cAMP elevates glucocorticoid receptor concentrations about twofold in hepatoma cells (*7*), we evaluated the effects of 8-Br cAMP on PR protein. Immunodots (Fig. 2C) showed that PR abundance was decreased about 60%, not increased, by 8-Br cAMP. Immunoblots (Fig. 2B) were performed to test the effects of 8-Br cAMP on receptor proteolysis. Because the amounts of PR produced in

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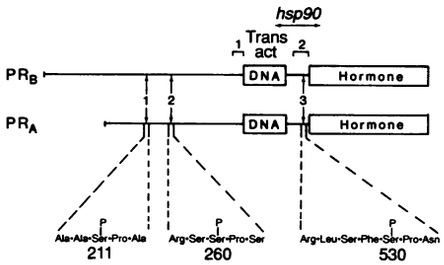

Fig. 1. Structural organization of PR_A and PR_B. The PR exists in two forms (PR_A and PR_B), each with a similar general structural organization. PR_B contains an additional 128 amino acids at the NH_2-terminus thought to be important for promoter- and tissue-specific regulation of transcription (*21*). Both PRs have identical hormone-binding and zinc finger DNA-binding domains. The sequence between these two domains contains one of two transactivation domains (*22*) and interacts with *hsp90* (*23*). At the NH_2-terminal side of the DNA binding domain is a second transactivation domain and regions that may allow the receptor to interact with other proteins (*21, 24*). DNA, zinc finger DNA-binding domain; hormone, progesterone-binding domain; trans act 1 and 2, transcriptional activation domains 1 and 2; *hsp90*, region of interaction with *hsp90*; 1, 2, and 3, location of in situ phosphorylation sites. The amino acid sequence (*5*) surrounding sites 1, 2, and 3 (Ser^{211}, Ser^{260}, and Ser^{530}) are shown at the bottom of the figure.

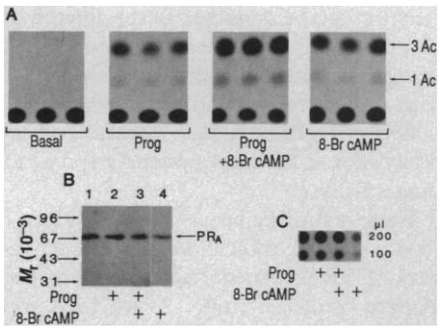

Fig. 2. Regulation of PR_A-mediated transcription and PR protein by progesterone and 8-Br cAMP in CV1 cells. (**A**) Transcriptional activity was measured in monkey kidney CV1 cells transfected by the polybrene method (*25*) with the PR_A expression plasmid (5 µg) and the reporter plasmid (10 µg), which contained a dimerized PRE fused to the herpes simplex virus thymidine kinase (*tk*) promoter and the bacterial *CAT* gene (PRE*tkCAT*) (*6, 9*). Cells grown in DMEM containing insulin, transferrin, and selenium (ITS; Collaborative Research) were untreated (basal) or treated with 10^{-7} M progesterone (prog), 10^{-3} M 8-Br cAMP, or progesterone plus 8-Br cAMP. Media was changed after 20 hours. The *CAT* activity was determined 42 hours after glycerol treatment (*26*). Arrows indicate the migration positions of the 1- and 3-acetylated forms of [^{14}C]chloramphenicol. The results are representative of at least six experiments where the induction ranged from 10- to 40-fold. (**B**) Receptor protein was studied by transfection of COS M6 cells with the PR_A expression plasmid (20 µg) by the calcium phosphate precipitation method (*22*). After 42 hours of the indicated treatments in DMEM containing ITS, high salt extracts were made and analyzed by immunoblotting as described (*3*) with PR22, a specific monoclonal antibody (provided by D. O. Toft). Values show the migration positions of standards with the indicated molecular weights. (**C**) Equal amounts of receptor protein from high salt extracts of CV1 cells were analyzed by immunoblotting (*3*).

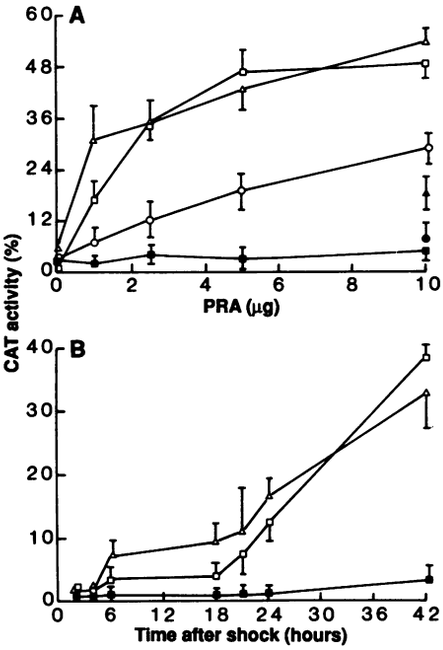

Fig. 3. Dependence of progesterone and 8-Br cAMP stimulated transcription on PR_A. (**A**) Cells were transfected with PRE*tkCAT* (10 µg) and the PR_A expression plasmid (10 µg) either with or without the PR_A sequences. Cells were untreated (filled squares), treated with progesterone (10^{-7} M) (open squares), or treated with 8-Br cAMP at concentrations of 10^{-3} M (open triangles), 10^{-4} M (open circles), 10^{-5} M (filled triangle), or 10^{-6} M (filled circle). After 42 hours of incubation, *CAT* activity was determined. These results are representative of at least three separate experiments and, as for all subsequent experiments, show the mean ± SD for triplicate plates. (**B**) Cells were transfected as in the legend to Fig. 2, and either untreated (filled squares), treated with 8-Br cAMP (10^{-3} M) (triangles), or treated with progesterone (10^{-7} M) (open squares). At the indicated times after glycerol treatment, cells were harvested and *CAT* activity was determined. The results are representative of two experiments.

CV1 cells are below the limit of detection for immunoblot analysis with our antibodies, we used COS M6 cells, a derivative of CV1 cells that express much higher concentrations of protein from transfected genes. Nearly equal amounts of intact, nonproteolyzed PRs were produced in each case. Thus, the transcriptional induction by 8-Br cAMP or progesterone was not a consequence of elevated PR protein concentrations or production of truncated, constitutively active PRs.

To determine whether the transcriptional response to 8-Br cAMP required the PR_A, we performed a dose-response analysis by transfecting CV1 cells with increasing amounts of PR_A expression plasmid (Fig. 3A). Induction by progesterone (10^{-7} M) was dependent on the amount of PR_A plasmid transfected. With 8-Br cAMP (10^{-3} M), a similar amount of induction was achieved. The response was less at lower doses of 8-Br cAMP and was absent when PR_A was omitted (PR_A transfected = 0 µg). These results show that 8-Br cAMP induction of transcription from PRE*tkCAT* [a dimerized PRE fused to the herpes simplex virus thymidine kinase (*tk*) promoter and the bacterial *CAT* gene] has an absolute requirement for PR_A. Furthermore, 8-Br cAMP did not stimulate basal transcription from PRE*tkCAT* in the absence of transfected PR_A, indicating that stimulation of PKA did not result in a general increase in transcriptional activity. In addition, while 8-Br cAMP induced PR_A-mediated transcription from PRE*tkCAT* 10- to 40-fold, maximal stimulation was less than 1.5-fold (*8*) for the following reporter genes, which lacked PREs in their regulatory regions: the herpes simplex virus *tk* promoter (*9*), the Rous sarcoma virus promoter (*10*), the SV40 promoter (*9*), and the human immunodeficiency virus type-1 (HIV-1) promoter (*11*).

The temporal onsets of transcriptional activation were similar for progesterone and 8-Br cAMP (Fig. 3B). These responses increased with time in parallel to the accumulation of PR_A (*8*), further supporting the observation that the transcriptional enhancement by 8-Br cAMP required PR_A.

Because stimulation of PKA mimicked progesterone action, we tested the hypothesis that inhibition of PKA blocks hormone action. A synthetic peptide that contains the active core region of the protein kinase inhibitor (PKI) is a potent and specific inhibitor of PKA (*12*). The peptide can enter cells, although to an unknown extent, thereby inhibiting intracellular PKA and the processes regulated by this kinase (*13*). We tested the PKI peptide for its ability to inhibit progesterone-induced gene expression in CV1 cells (Fig. 4). At an extracellular concentration of 10 µg/ml, the peptide antagonized progesterone-dependent activation of PR_A-mediated transcription with little effect on basal activity. H8, a less specific inhibitor of PKA (*14*), also blocked hormone action in a dose-dependent manner with little effect on basal activity.

We next tested whether inhibition of protein phosphatases could enhance PR-mediated transcription. Transfected CV1 cells were treated with okadaic acid, a potent inhibitor of protein phosphatases 1 (PP1) and 2A (PP2A) (*15*). These phosphatases are the predominant cellular protein phosphatases and act on a wide range of serine

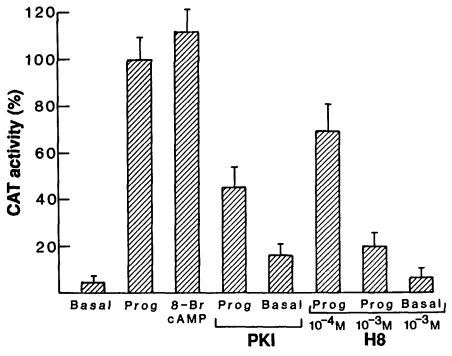

Fig. 4. Regulation of PR_A-mediated transcription by modulators of PKA. CV1 cells were transfected as described in the legend to Fig. 2 and either untreated (basal) or treated with progesterone (10^{-7} M) (prog), 8-Br cAMP (10^{-3} M), PKI (10 μg/ml) in the presence (prog) or absence (basal) of progesterone (10^{-7} M), or H8, at the concentrations shown, in the presence (prog) or absence (basal) of progesterone (10^{-7} M). After 42 hours, *CAT* activity was determined. The results are representative of at least two experiments and were normalized to progesterone-stimulated *CAT* activity (35% conversion of substrate).

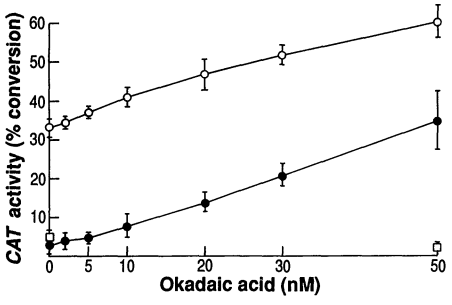

Fig. 5. Regulation of PR_A-mediated transcription by inhibition of protein phosphatases. Cells were treated with the indicated concentrations of okadaic acid in the absence (closed circles) or presence (open circles) of progesterone (10^{-7} M). Control cells were transfected with an expression plasmid that did not contain PR_A sequences (open squares). The results are representative of at least six experiments.

and threonine phosphoproteins in vivo (*16*). Okadaic acid activated transcription in a dose-dependent manner similar to that observed with progesterone (Fig. 5). In the absence of PR_A, okadaic acid had no effect on basal transcription, indicating that transcriptional enhancement by okadaic acid required PR_A and was not the result of a general, nonspecific activation of transcription. Maximal activity occurred at about 50 nM okadaic acid with higher concentrations substantially inhibiting cell growth over the 2 days of treatment. The okadaic acid response was increased in the presence of progesterone, which is similar to the additive effects observed with 8-Br cAMP and progesterone (Fig. 3).

From the present work, we conclude that progesterone-dependent, PR-mediated transcription can be mimicked in the absence of progesterone by pharmacological modulation of cellular protein kinases and phosphatases. That progesterone action can be blocked by inhibitors of PKA further implicates phosphorylation in the transcriptional response to progesterone.

These and other results (*2, 3, 5*) suggest several possible mechanisms for the activation of PR-mediated transcription in the absence of progesterone. As we have presented no evidence for PR phosphorylation in CV1 cells, activation could occur by phosphorylation of the PR or other proteins of transcription complexes. Highly purified PR_A is phosphorylated in vitro by PKA at Ser^{528} near the site (Ser^{530}) of progesterone-induced phosphorylation (*5*). These sites are in one of the PR transactivation domains, and raise the possibility that in the present experiments PKA-mediated phosphorylation of Ser^{528} substituted for phosphorylation of Ser^{530} by providing a negative charge required for transactivation (*17*). However, this does not explain the block of hormone action by inhibitors of PKA.

A more likely possibility is that PKA activates PR-mediated transcription by phosphorylation, via a cascade mechanism, of another kinase or phosphatase. This ultimately would result in phosphorylation of the PR or other transcription regulatory proteins, or both. Kinases and phosphatases are known to interact in such complex relations; for example, activation of PKA or inhibition of PP2A results in inhibition of PP1 (*18*). Thus, since many of the substrates of these enzymes are themselves kinases or phosphatases, both feed-back and feed-forward (cascade) mechanisms are operative. The present studies support the hypothesis that phosphorylation regulates PR-mediated transcription, but the kinases and phosphatases directly involved in progesterone action in vivo remain unidentified.

On the basis of the results presented in this report, we suggest a mechanism for PR action in which an equilibrium, regulated by kinases and phosphatases, exists between transcriptionally active and inactive receptors. In the absence of progesterone, the PR is a poor substrate for kinases or a good substrate for phosphatases and is transcriptionally inactive. In the presence of the hormone, a conformational change occurs in the receptor that results in a net increase in phosphorylation. Once this has occurred, the receptor is transcriptionally active and has a reduced requirement for ligand occupancy.

These observations are not restricted to the chick PR_A. In the absence of progesterone, 8-Br cAMP and okadaic acid activate transcription of PR_B and of a chimeric construct coding for a fusion protein of the COUP (chicken ovalbumin upstream promoter) transcription factor (*19*) and the PR DNA-binding domain (*20*). Thus, ligand-induced conformational alterations and subsequent receptor phosphorylation may be important for conversion of members of the receptor superfamily to the transcriptionally active state in vivo. Finally, our studies show that progesterone occupancy of the receptor is not required for the formation or function of an active transcription complex.

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20 June 1990; accepted 24 September 1990

Early Enhancement of Calcium Currents by H-*ras* Oncoproteins Injected into *Hermissenda* Neurons

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Influx of calcium through membrane channels is an important initial step in signal transduction of growth signals. Therefore, the effects of Ras protein injection on calcium currents across the soma membrane of an identified neuron of the snail *Hermissenda* were examined. With the use of these post-mitotic cells, a voltage-sensitive, inward calcium current was increased 10 to 20 minutes after Harvey-*ras* oncoproteins were injected. The effects of oncogenic Harvey *ras* p21 protein (v-Ras) occurred quickly and were sustained, whereas the effects of proto-oncogenic *ras* protein (c-Ras) were transient. This relative potency is consistent with the activities of these oncoproteins in stimulating cell proliferation. Thus, this calcium channel may be a target for Ras action.

THE *ras* PROTO-ONCOGENES (c-*ras*) encode highly conserved, membrane-associated low molecular weight (21,000) proteins with guanosine triphosphatase (GTPase) activity, which serve a fundamental, but as yet poorly defined, function in the control of cell growth and development (*1*). Genes with point mutations encode modified proteins (an oncogenic form of Ras, v-Ras) with impaired GTPase activity, which inhibits their normal shut-off mechanism and causes sustained activation of unknown Ras targets, which probably leads to oncogenic transformation. Intracellular injection of c-Ras or v-Ras into certain quiescent cell lines induces pinocytosis and membrane ruffling (*2*) and, eventually, cell growth or transformation (*3*). The earliest reported effect after injection of Ras is an increase in pH within 10 min of injection with v-Ras but not c-Ras. This effect was attributed to a stimulation of the Na^+/H^+ antiporter (*4*). Activation of phosphoinositide metabolism is also induced rapidly after injection of Ras (*1*), but this effect occurs with other oncogenes as well (*5*). Ras proteins are also involved in cellular growth induced by platelet-derived growth factor (PDGF) or epithelial growth factor (EGF) (*6*). Activation of EGF receptors induces early intracellular events similar to those induced by Ras injection: stimulation of phospholipid metabolism and an increase in intracellular pH, as well as transient increases in intracellular Ca^{2+} concentration (*7*). In fact, Ca^{2+}-free extracellular medium inhibited Ras-mediated pinocytosis and membrane ruffling in NIH 3T3 cells (*2*), suggesting that Ca^{2+} influx through membrane channels may be a crucial initial step in Ras transduction of growth signals. Ca^{2+} and Na^+ (*8*) channels are down-regulated in cell lines transformed by Ras. Because proto-oncogenes, including *ras*, are also expressed in nervous tissue and they have been suggested to have a role in neural signaling and modification (*9–11*), we studied the effects of injections of Ras on voltage-sensitive inward Ca^{2+} currents in a fully differentiated, quiescent neuron from *Hermissenda*.

Under two-electrode voltage clamp of the giant left pedal 1 neuron LP_1 (150 to 200 μm diameter), we isolated a sustained voltage-dependent inward current carried by Ca^{2+}. This Ca^{2+} current was maximally activated by positive steps to 10 mV, from a holding potential of −60 mV, but it could also be activated by less positive commands (for example, to −40 mV) from more negative holding potentials (for example, −100 mV). Such characteristics are similar to those of *Aplysia* neurons (*12*). The Ca^{2+} current inactivated with a very long time constant (>500 ms) and was blocked by external application of cadmium or nifedipine, ruling out the possibility that it was due to a covert K^+ outward current or other inward current. Typical recordings are shown in Fig. 1. For our experiments, the inward current was elicited by depolarizing steps to 0 mV before and after iontophoretic injections of v-Ras and c-Ras, with 10 mM Ba^{2+} as the charge carrier. By pressure-injecting different concentrations of Ras into LP_1 (2 bar, 0.1 Hz, 50-ms pulses), we determined that the minimum concentration required to detect any effect on the Ca^{2+} currents was 150 μg/ml and 300 μg/ml for v-Ras and c-Ras, respectively. Because an increased leakage conductance and a rundown in Ca^{2+} conductance of more than 20% was observed 20 min after pressure-injection, iontophoresis with negative current pulses (−10 nA, 10 min) was sub-

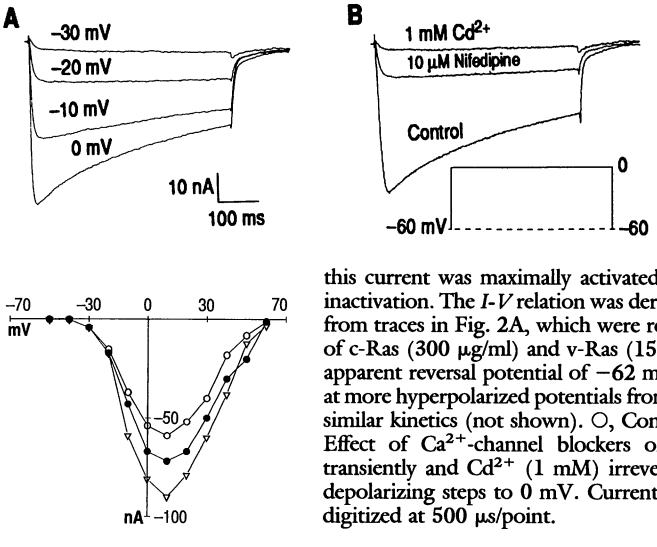

Fig. 1. Typical voltage-clamp recordings (*22*) of voltage-sensitive inward Ca^{2+} currents (I_{Ca}) from *Hermissenda* LP_1 neurons. Ba^{2+} (10 mM) substituted for Ca^{2+}, and there was 3 M $CsCl_2$ in the microelectrodes. (**A**) Current-voltage (*I-V*) relation from a −60 mV holding potential (V_H); this current was maximally activated at 10 mV and showed little inactivation. The *I-V* relation was derived from the traces above and from traces in Fig. 2A, which were recorded 30 min after injection of c-Ras (300 μg/ml) and v-Ras (150 μg/ml). The current had an apparent reversal potential of −62 mV and could also be activated at more hyperpolarized potentials from a V_H of −100 mV with very similar kinetics (not shown). ○, Control; ●, c-Ras; ▽, v-Ras. (**B**) Effect of Ca^{2+}-channel blockers on I_{Ca}. Nifedipine (10 μM) transiently and Cd^{2+} (1 mM) irreversibly reduced I_{Ca} elicited by depolarizing steps to 0 mV. Current transients in this figure were digitized at 500 μs/point.

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