value of the octopamine agonist for inhibition of leaf consumption) was DDCDM + IBMX = NC7 + IBMX-octopamine + IBMX = DDCDM alone > NC7 alone > octopamine alone (almost inactive). Thus, pesticidal effectiveness of various octopamine agonists, alone or combined with IBMX, agreed well with the ability of these same agents, alone or in combination, to increase tissue cyclic AMP (Table 1).

These data, together with the following additional observations, provide supporting evidence for an involvement of cyclic AMP in the primary and synergistic pesticidal effects of the methylxanthines. (i) Forskolin, a diterpene that appears to activate the catalytic subunit of adenylate cyclase directly (*19*), stimulated cyclic AMP production in hornworm nerve cord in the absence of calcium and, in leaf-eating experiments, caused a disruption of feeding that was enhanced by IBMX. (ii) IBMX did not enhance the pesticidal effects of certain insecticides, including DDT (a chlorinated hydrocarbon), chlorpyriphos (organophosphate), and Karathane, none of which stimulated adenylate cyclase activity in insects in vitro. (iii) The meta-hydroxy isomer of octopamine was at least ten times less potent than octopamine (where the hydroxyl is in the para position) in activating octopamine-sensitive adenylate cyclase in insects; likewise, in the presence of IBMX, the meta isomer of octopamine showed no pestistatic or pesticidal activity whereas octopamine did. (iv) Among several phenyliminoimidazolidine derivatives, there was a rank-order correlation between the ability to activate adenylate cyclase in hornworms and the ability to disrupt their feeding on tomato leaves. (v) Finally, lipid-soluble cyclic AMP analogs such as the *p*-chlorophenylthio derivative of cyclic AMP were observed to disrupt feeding. These analogs, which were found in vitro to undergo significant hydrolysis by PDE activity in nerve cord (an effect blocked by IBMX), had their antifeeding activity in vivo enhanced by IBMX.

Taken together, these data suggest that the pestistatic and pesticidal effects of the methylxanthines are mediated through an alteration of concentrations of cyclic AMP in tissue, most likely secondarily to an inhibition of PDE. These findings also suggest that naturally occurring methylxanthines could function as endogenous insecticides. Finally, these results raise the possibility that methylxanthines or nonxanthine PDE inhibitors may be of some practical use in pest control, either alone or as synergists of certain other primary pesticides. In the latter case, potential toxicity for mammals could be reduced by targeting the primary pesticide at a receptor-associated adenylate cyclase (for example, octopamine) found primarily or exclusively in invertebrates (*20, 21*).

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Regulation of a Hybrid Gene by Glucose and Temperature in Hamster Fibroblasts

Abstract. *A novel eukaryotic hybrid gene has been constructed from the 5' sequence of a rat gene and the bacterial neomycin-resistance gene. After transfection into hamster fibroblasts, the neo transcripts can be induced to high levels by the absence of glucose. Furthermore, this hybrid gene can be regulated by temperature when it is introduced into a temperature-sensitive mutant cell line.*

The ability to introduce defined DNA segments into mammalian cells is a powerful tool for studying the regulation of eukaryotic gene expression and for identifying specific DNA sequences responsible for such regulation. We have been using DNA-mediated gene transfer techniques to study the regulated expression of a set of "glucose-regulated proteins" (GRP's) in mammalian cells. The GRP's are cellular proteins synthesized constitutively at low but detectable levels under normal tissue culture conditions or in whole organs; yet their synthesis is markedly enhanced in response to glucose starvation or exposure to inhibitors of glycosylation (*1*). The most abundant GRP in chicken, hamster, rat, mouse, and human cells is a 78-kilodalton protein. While a one- to twofold increase in GRP78 can be detected after heat shock of the cultured cells, it is distinct from the major 72- to 73-kilodalton heat shock protein commonly observed in mammalian cells (*1, 2*).

We and others have described a temperature-sensitive (ts) hamster mutant cell line, K12, which overproduces the GRP's by a factor of 20 to 50 when the cells are incubated at the nonpermissive temperature, 40.5°C (*3*). Since the messenger RNA (mRNA) for GRP78 is also

overproduced in this *ts* mutant cell line at 40.5°C, the K12 system has provided the opportunity for the molecular cloning of the gene encoding the GRP78 (*4*) and the characterization of the mRNA synthesis induced either by the temperature shift (*5*) or by glucose starvation (*6*). Thus, in the previous studies, we obtained a complementary DNA (cDNA) plasmid, p3C5, which by the criterion of hybrid-select translation (*4*) coded for GRP78. Further, we demonstrated that, under normal conditions, the transcripts encoded by p3C5 comprised about 0.1 percent of cytoplasmic polyadenylated [poly(A)$^+$] RNA, implying that its regulator-promoter sequence is moderately active under normal conditions. However, when the cells were deprived of glucose at 35°C or shifted to 40.5°C in normal culture medium, there was a rapid increase in its transcription rate so that at steady state the p3C5 transcripts constituted about 1 percent of the cytoplasmic poly(A)$^+$ RNA.

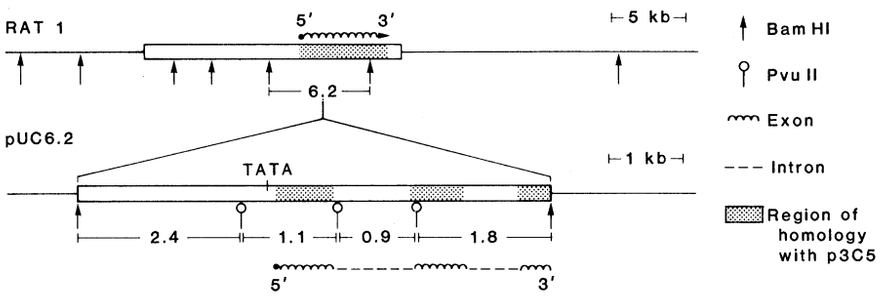

Fig. 1. Schematic diagram of the rat genomic clone RAT 1. RAT 1 was isolated from a partial Eco RI rat genomic library cloned in Charon 4A (*7*). RAT 1 contains about 17 kb of rat genomic sequence. The 5′ region of the RAT 1 gene was defined further by subcloning a 6.2-kb Bam HI fragment into the Bam HI site of pUC8 (*8*). The restriction enzyme Pvu II cleaved the 6.2-kb rat insert into four fragments of length 2.4, 1.8, 1.1, and 0.9 kb. Fine restriction mapping and hybridization of these subfragments with p3C5 and cDNA probes made from rat mRNA showed that the RAT 1 transcriptional unit initiated about 2.8 kb from the left Bam HI site. DNA sequence analysis of the 1.1-kb Pvu II fragment revealed the presence of a CCAAT (C, cytosine) sequence and a TATATAA sequence 5′ to the proposed transcription initiation site. Since both the 0.9- and 1.8-kb Pvu II fragments contain sequences unrelated to p3C5, it is likely that at least part of these fragments contain rat-specific intervening sequences. The direction of transcription and the appropriate locations of the exons and introns are indicated. The total length of the RAT 1 mRNA represented in this 6.2-kb fragment is estimated to be 2 kb.

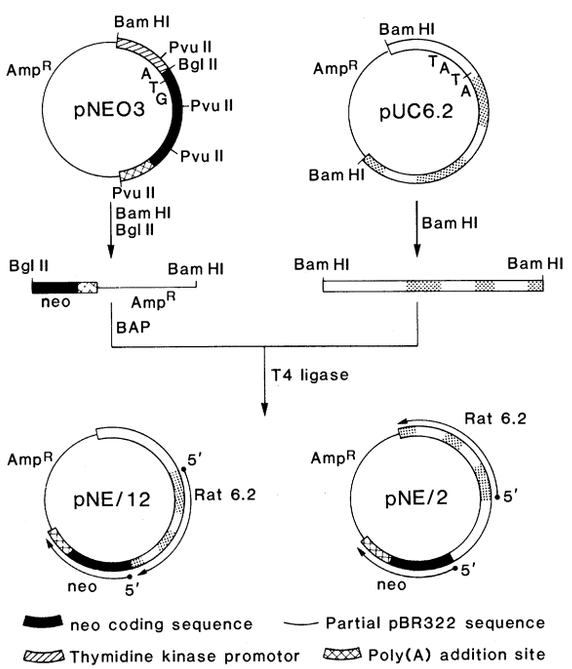

Fig. 2. Construction of the RAT 6.2–neo hybrid genes. The plasmid pNEO3 was digested with Bam HI and Bgl II, yielding a 4.4-kb fragment that contained the entire neo coding sequence and partial pBR322 sequence, including the origin of replication and the β-lactamase gene, and a 0.7-kb fragment that contained the thymidine kinase promoter sequence from herpesvirus. The 4.4-kb fragment was separated from the 0.7-kb fragment by gel electrophoresis and recovered from low-melting agarose gels. Similarly, the 6.2-kb Bam HI fragment containing the RAT 1 gene was recovered from pUC6.2 by Bam HI treatment and electrophoresis. To reduce self-ligation of the vector, we treated the 4.4-kb Bam HI–Bgl II fragment with bacterial alkaline phosphatase before ligation with the 6.2-kb RAT 1 DNA. The ligated mixture was transfected into HB101, and ampicillin-resistant colonies were selected. The orientation of insertion of the 6.2-kb RAT 1 DNA insert with respect to the neo gene was determined by restriction analysis of the hybrid gene. In the plasmid designated pNE/12, the orientation of transcription for the RAT 1 gene was the same as that of the neo gene, which is 1.2 kb in length. If the RNA initiates at the RAT 1 promoter, splices properly, and terminates at the poly(A) addition site downstream from the neo gene, the size of the RNA transcribed from the hybrid gene is expected to be 3 to 3.5 kilobases. In pNE/2, the orientation of the RAT 1 sequence is opposite that of the neo sequence.

In order to determine whether the flanking sequence of this gene contained the regulatory signals required for its induction in glucose-starved cells and its specific induction in the mutant K12 cells at 40.5°C, we isolated rat genomic clones according to whether they cross-hybridized with the hamster p3C5 cDNA sequence (*7*). One genomic clone, designated RAT 1, contained within its 17-kilobase insert the entire p3C5 sequence and its 5′ and 3′ flanking sequences. A partial restriction map of RAT 1 is shown in Fig. 1. A 6.2-kb Bam HI fragment that contained the 5′ sequence of the rat gene was subcloned into the plasmid vector pUC8 (*8*) for fine restriction mapping and cross hybridization with p3C5 and 3′ selected hamster and rat cDNA probes. The results of these hybridizations allowed us to orient the direction of transcription as shown in Fig. 1 and suggested that this fragment contains about 2.8 kb of the 5′ flanking sequence of the rat gene. The initiation site for transcription was further confirmed by primer extension analysis, and the location of the TATA (T, thymine; A, adenine) sequence was determined by DNA sequencing (*9*).

To test whether the 6.2-kb Bam HI sequence contained a functional promoter or the regulatory sequence responsible for the enhanced transcription in glucose-starved cells (or both), we constructed hybrid genes with the 5′ sequence of the rat glucose-regulated gene fused to the bacterial phosphotransferase gene (neo) that confers resistance to the neomycin-kanamycin antibiotic G418, which is normally toxic to diverse animal cells (*10*). For this purpose we removed the thymidine kinase promoter of herpesvirus from plasmid pNEO3 (*11*), and in its place introduced the rat 6.2-kb Bam HI fragment in both orientations. The scheme for the construction of the hybrid genes is shown in Fig. 2. Unlike other neo transfection vectors commonly used (*12*), the constructs described here do not contain any SV40 enhancer or promoter sequences. Thus, the only eukaryotic sequences present in these hybrid genes are the rat DNA and the short stretch of poly(A) addition site (derived from the thymidine kinase gene of the herpesvirus) which was fused to the 3′ end of neo in the original pNEO3 construct.

First, we determined the transformation efficiencies of the hybrid genes in hamster fibroblasts. The RAT 6.2-neo hybrid genes were transfected into the

nonmutant, parental cell line Wg1A, as well as K12, the *ts* mutant. Stable transformants were selected on the basis of their resistance to antibiotic G418. We demonstrated earlier that these fibroblast cell lines are highly competent for DNA-mediated gene transfer when pNEO3 and the calcium phosphate precipitation technique are used (*13*). The antiobiotic G418 was toxic to these cells at concentrations of 300 to 450 μg/ml, and the frequency of cells surviving at these G418 concentrations was about 2×10^{-7}. We observed that when the rat 6.2-kb fragment and the neo structural gene were fused in the same orientation of transcription, the efficiency of transformation of the hybrid gene was one-fifth to one-third that of the plasmid pNEO3. However, if the rat sequence was fused to the neo gene in the opposite transcriptional orientation, the transformation efficiency was about 1/30 that in both K12 and Wg1A cells (data not shown).

To further characterize the expression of the hybrid genes after transfection, individual stable transformants from Wg1A and K12 were picked and expanded for the analysis of the neo mRNA concentration under various culture conditions. In this set of experiments, the nonpermissive temperature used for the K12 cells was 39.5°C (*14*). Total cytoplasmic RNA was prepared from the cells and equal amounts (10 μg) of RNA were applied to the denaturing formaldehyde-formamide agarose gels. The hybridization probe used for the detection of the neo mRNA was a combination of three Pvu II fragments (0.76, 0.72, and 0.51 kb) from pNEO3 that contain the Tn5 neo sequence (Fig. 2). Examples of the RNA blot hybridization results from the stable transformants tested are shown in Fig. 3.

First we examined the few transformants isolated by transfecting K12 cells with pNE/2, the hybrid gene containing RAT 6.2 and neo in opposite orientations. Two patterns of neo transcripts were observed. As shown in Fig. 3A, in pNE/2-1, there were no detectable neo transcripts, suggesting that these cells were either statistical survivors or variants of K12 that developed resistance to G418. In pNE/2-2, there was a detectable level of neo mRNA. However, the level of neo mRNA remained the same at both 35° and 39.5°C.

Next we analyzed the neo mRNA levels in K12 transformants that had integrated into their chromosomes two to five copies of pNE/12, the hybrid gene containing the same direction of transcription for the RAT 6.2 and neo gene (Fig. 3B). We examined four stable transformants: all four showed a five- to tenfold increase in neo mRNA levels upon shifting to 39.5°C in regular medium containing glucose. The increase in neo mRNA was similar in magnitude to that of the induction of the endogenous p3C5 transcript in the K12 recipient cells (*5*). Since we showed earlier that the p3C5 transcript was not responsive to treatment with a glucocorticoid such as dexamethasone but could be induced to high levels by glucose starvation (*5, 6*), we tested whether the hybrid gene would respond similarly. The stable transformant pNE/12-3 was treated with dibutyryl adenosine 3′,5′-monophosphate (cyclic AMP) and dexamethasone or glucose-free medium; the level of neo transcripts in the cells was then determined (Fig. 3C). As in the case of the p3C5 transcripts, the neo transcripts were specifically induced either by glucose starvation or by shifting to 39.5°C in medium containing glucose. Actinomycin D, an inhibitor of transcription, eliminated this response.

An interesting question then arises: would the expression of the hybrid genes be regulated differently in the nonmutant fibroblast cells? Although the p3C5 transcripts can be induced rapidly to high levels in both K12 and WglA cells in culture medium deprived of glucose, we have observed that p3C5 transcripts are increased by a factor of only 1.5 to 2 in nonmutant hamster fibroblasts at 40.5°C (*15*). This finding is consistent with the

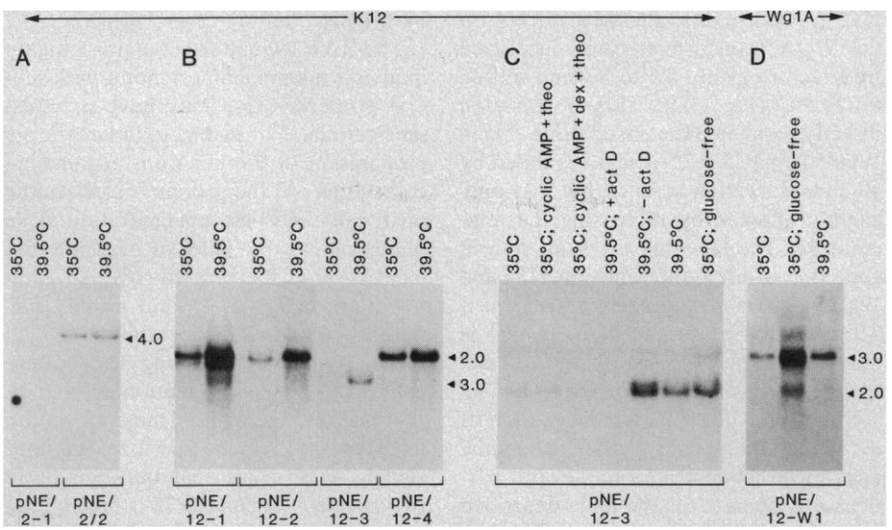

Fig. 3. Level of neo mRNA in stable transformants. The conditions for DNA transfection were as described (*13*). Two days after the transfection, G418 was added to attain a concentration of 400 μg/ml. Individual G418-resistant colonies were picked after 4 weeks and expanded into mass cultures. For the extraction of total cytoplasmic RNA, the transformants were seeded in 150-mm-diameter culture dishes and grown to 90 percent confluency in Dulbecco's minimal essential medium (DMEM). At this time, parallel sets of cells were subjected to control and different culture conditions. At the end of treatment, total cytoplasmic RNA was extracted from the cells (*5*); 10 μg of each RNA sample was applied to formamide-formaldehyde agarose gels and, after electrophoresis, was blotted on nitrocellulose filters. The RNA gel blots were hybridized with nick-translated neo DNA fragments (0.76-, 0.72-, and 0.51-kb fragments derived from Pvu II digestion of pNEO3). The specific activity of the neo probes ranged from 2×10^8 to 4×10^8 count/min per microgram of DNA. Conditions for the RNA blot and hybridization have been described (*5, 6*). (A) pNE/2-1 and pNE/2-2 were two independent transformants derived from K12 cells transfected with pNE/2. RNA was extracted from cells incubated for 16 hours at 35°C or 39.5°C in DMEM. (B) pNE/12-1, pNE/12-2, pNE/12-3, and pNE/12-4 were four independent transformants derived from transfecting K12 cells with pNE/12. RNA was extracted from the cells incubated for 16 hours at 35° or 39.5°C in DMEM. (C) RNA was extracted from pNE/12-3. The culture conditions for each sample lane was as follows: 16 hours of incubation at 35°C in DMEM; 35°C in DMEM supplemented with 40 m*M* dibutyryl cyclic AMP and 40 m*M* theophylline; 35°C in DMEM supplemented with 1mM dexamethasone in addition to dibutyryl cyclic AMP and theophylline; 4 hours at 39.5°C in DMEM with actinomycin D after the cells were treated with actinomycin D (4.5 μg/ml) for 2 hours at 35°C; 4 hours at 39.5°C in DMEM; 16 hours at 39.5°C in DMEM; and 16 hours at 35°C in glucose-free medium (*6*). (D) pNE/12-W1 was a stable transformant derived from transfecting Wg1A with pNE/12. RNA was extracted from cells after 16 hours of incubation at 35°C in DMEM; 16 hours at 35°C in glucose-free medium, and 16 hours at 39.5°C in DMEM. The sizes (in kilobases) of the neo transcripts are indicated. Of ten pNE/12 transformants analyzed in K12 and Wg1A, eight exhibited a neo transcript of about 3 kb, which is the size expected if the RNA initiated at the RAT 1 promoter was properly spliced, and terminated at the thymidine kinase poly(A) addition site. The remaining two pNE/12 transformants exhibited a neo transcript size of 2 kb, as is demonstrated in pNE/12-3. Both types of transcripts were inducible by glucose starvation and incubation at 39.5°C.

observation that GRP78 is only slightly heat-inducible in WglA cells and with the suggestion that a recessive *ts* mutation in K12 cells causes a large increase in GRP78 as well as the transcription of p3C5 mRNA at 39.5°C (*3*). One hypothesis is that in nonmutant fibroblasts such as WglA, a *trans*-acting factor is effectively regulating the transcription of the p3C5 gene at a basal level at both 35°C and 39.5°C; whereas in K12, this factor is inactivated at 39.5°C because of the *ts* mutation. To test the existence of this *trans*-acting factor in normal fibroblasts, we transfected WglA cells with the hybrid genes and measured the neo mRNA levels in stable transformants. Of the five transformants tested that contained pNE/12, three showed a 3- to 10-fold increase in neo mRNA under glucose starvation conditions. In contrast to the K12 transformants, the neo mRNA in the WglA transformants was increased by a factor of only 1.5 to 2 when shifted to 39.5°C (Fig. 3D). This observation directly paralleled the observation that in WglA the p3C5 mRNA was regulated by glucose starvation at 35°C, but was only slightly affected by increasing the temperature. The fact that the same hybrid gene is regulated differently in K12 and WglA at 39.5°C suggests that a *trans*-acting factor present inside the cell is modulating the response of the *cis* element present on the rat genomic DNA.

Studies of the regulated expression of inducible eukaryotic genes in animal cells have concentrated on several particular systems (*16*). We have described a novel inducible eukaryotic gene system that is regulated by the availability of glucose in the culture medium. In addition, this gene is also regulated by temperature in the *ts* mutant cell line K12. This suggests that the rat genomic sequence we isolated contains a putative control region that responds to glucose starvation as well as to the K12 *ts* mutation. Deletion analysis of the rat genomic DNA coupled with site-specific mutagenesis of the hybrid genes will allow us to further define the control sequence and to differentiate between single or separate regulatory sites for these two responses. In any case, the results we obtained using hybrid gene constructs with two different transcriptional orientations seem to indicate that the regulator-promoter sequence was functional only when it was fused in the same orientation as the downstream structural gene. We have also inserted the 6.2-kb Bam HI fragments of the RAT 1 sequence in both orientations into the single Bam HI site of another selectable marker, pSV2gpt (*17*), which contains the SV40 enhancer sequence; stable transformants were obtained (*13*). Using the pBR322 sequence downstream from the rat insert as a probe, we were able to detect discrete transcripts that initiated at the rat promoter and terminated in the pBR322 sequence. These transcripts were specifically inducible in K12 transformants at 39.5°C, as were the neo transformants (*15*). Therefore, the SV40 enhancer sequence linked several kilobases upstream or downstream from the RAT 1 sequence did not seem to affect the regulatory response in these fibroblast cells.

The RAT 1 sequence contains a highly inducible mammalian promoter with several properties that may have practical applications. For instance, in large-scale preparation of protein from mammalian cell cultures, if the protein is toxic to the host cells in high concentration it is important to be able to regulate the expression of the protein (*18*). An additional advantage of this rat promoter is that it can activate transcription to high levels by simply starving the culture cells. The proper regulation of the rat genomic sequence after transfection into hamster cells showed that the regulatory signals were conserved between these two species. Since GRP78 is highly conserved in animal cells (*4*), it is likely that this promoter will be functional in various recipient cells.

Finally, the transformants we obtained were apparently producing a functional phosphotransferase protein in order to overcome the G418 toxicity. It will be interesting to determine in these transformants whether the neomycin-resistance activity was derived from the synthesis of a fusion RAT-neo protein or the utilization of the initial initiation codon from the neo gene itself, several recent reports having implied that such a process was feasible (*19*).

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