Precocene unequivocally inhibits or stops allatal development at any stage of application since precocene treatment can both prevent JH secretion when applied to newly eclosed females, or it can stop allatal production of JH if applied to sexually mature females. The fact that functional corpora allata can be stopped from secreting JH explains the precocious metamorphosis of precocene-treated nymphal stages.

These studies confirm that the size of the corpora allata is linked to JH secretion and that the ovaries are dependent upon JH for development, and show that the ultimate action of precocene prevents allatal growth and JH secretion. Since the ovaries of precocene-sterilized insects remain fully poised to respond to exogenous JH, it appears that precocene has no direct action upon the ovary.

Since precocene stops JH secretion through inhibition of allatal development, we suspect that precocene interferes with humoral or nervous regulation of the corpora allata. For the precocenes, therefore, we suggest the term antiallatotropin rather than anti-juvenile hormone since the latter term is more general and could encompass other modes of hormone antagonism including interference with JH biosynthesis, transport, or action at a receptor site.

Although these studies show that precocene prevents JH secretion by inhibiting allatal development, we have not demonstrated whether precocene acts directly upon the corpora allata or whether its action is mediated through some other organ that is its actual target. Numerous investigations have established the brain as the prime regulator of the corpora allata (*6*), and therefore the brain may be the actual target of precocene. Should this be the case, it may be possible to utilize precocene in the investigation of brain-allatal interactions and the development of new insect-growth regulators.

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Expression of Murine Sarcoma Virus Genes in Uninfected Rat Cells Subjected to Anaerobic Stress

Abstract. *Exposure of uninfected rat cells in tissue culture to anaerobic culture conditions induces transcription of RNA corresponding to the two principal constituents of rat-derived type-C sarcoma virus genomes: (i) those specific rat cell sequences present in the Kirsten and Harvey murine sarcoma virus genomes, and (ii) an endogenous type-C rat leukemia virus.*

Increasing evidence in both murine and avian systems indicates that the genomes of the highly oncogenic type-C sarcoma viruses are composed of normal cellular genetic information, recombined in an abnormal form. A major portion of the sarcoma virus genomes represents type-C leukemia viruses, which in turn have been shown to exist in a latent but inducible form within normal cell DNA (*1*). Such leukemia viruses are only weakly oncogenic in that they have long latent periods for tumor induction in vivo and generally fail to transform cells in culture (*2*). Specific additional cellular RNA sequences recombined with leukemia virus genes have been identified in sarcoma virus genomes (*3*, *4*). Since type-C sarcoma viruses readily transform cultured cells, quickly induce tumors in vivo, and carry at least one defined transforming gene (*5*), characterization of sarcoma virus gene function offers a reasonable hope of explaining how viruses can directly induce cancer. We report here studies aimed at elucidating sarcoma virus gene function, particularly by identifying those variables which influence the expression by normal cells of sarcoma virus genome components.

The Kirsten and Harvey murine sarcoma viruses represent recombinants between the type-C mouse leukemia viruses with which each was first isolated (Kirsten murine leukemia virus and Moloney murine leukemia virus) and the same set of specific rat cellular genetic information (*3*, *6*). With techniques described elsewhere (*3*), we have used a [^{3}H]thymidine-labeled reverse transcrip-

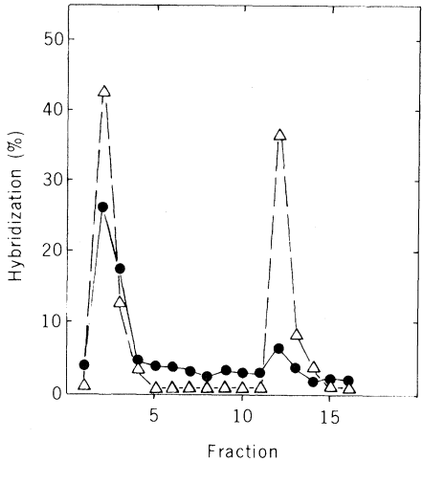

Fig. 1. Oligo(T) cellulose column chromatography of Fischer rat cell RNA. RNA (0.8 mg) was loaded on a 2.2-ml column containing 0.7 g of oligo(T) cellulose (Collaborative Research, Waltham, Mass.), according to the procedure of Scolnick *et al.* (*19*). The nonadsorbed material was removed by elution with 0.5*M* KCl, 0.01*M* tris, *p*H 7.5, in ten 2-ml fractions. The bound material was eluted in 0.01*M* tris, *p*H 7.5, in six 2-ml fractions. After monitoring the absorption of each fraction at 260 nm (98.6 percent of the input RNA eluted in the void, 1.4 percent was adsorbed on the column), 300 μg of yeast transfer RNA and two volumes of ethanol were added to each fraction. The precipitated RNA was resuspended in 100 μl of 0.1*M* NaCl, 0.01*M* tris, *p*H 7.5, and 0.001*M* EDTA. ^{3}H-Labeled DNA products of the Kirsten murine sarcoma virus (KiMSV) and the Wistar-Furth (WFU) rat leukemia virus were prepared and used as molecular hybridization probes as described elsewhere (*3*). Hybridization to WFU-RaLV [^{3}H]DNA is expressed directly, while that to KiMSV (KiMuLV) [^{3}H]DNA is normalized to its rat-specific fraction (40 percent). Hybridization was carried out in a 50-μl reaction mixture containing [^{3}H]DNA (2×10^3 count/min; 0.3 ng) and 20-μl resuspended column fractions in a buffer composed of 38 percent formamide, 600 m*M* NaCl, 50 m*M* 2-{[tris(hydroxymethyl)]-methylamino}ethanesulfonic acid (TES), *p*H 7.5, and 1 m*M* EDTA. After incubation for 7 days at 43°C, hybridization was monitored with nuclease *S*1. Increased quantities of oligo(T) cellulose column fractions 2 and 12 were able to hybridize over 90 percent of each [^{3}H]DNA. Plain cellulose [without coupled oligo(T)] gave no retention of either RNA species. Symbols: ●, hybridization to WFU-RaLV [^{3}H]DNA; △, hybridization to the rat-specific fraction of the [^{3}H]DNA product of KiMSV (KiMuLV).

Table 1. Expression of MSV-rat or RaLV RNA by Fischer rat embryo cells in culture. Sparse cultures of Fischer rat cells were established in roller bottles 3 days before treatment, and were fed with Dulbecco's modified Eagle's medium (Gibco) containing 10 percent calf serum every 24 hours. While subconfluent, the cultures were exposed for 48 hours to the agents indicated, and then total cell RNA was prepared as described elsewhere (*3*). Concentrations of the two RNA species relative to the levels in untreated cells were determined by molecular hybridization of at least four different amounts of cell RNA, as described in Fig. 1, followed by measurement of the displacement of the C_0t curves.

Treatment	Drug concentration (μg/ml)	Relative concentration of specific RNA sequences	
		MSV-rat	RaLV
Fischer rat embryo cells, untreated	0	1.0	1.0
Respiratory blocks			
Anaerobic growth	0	110	160
Dinitrophenol	18.4	1.0	1.1
Oligomycin	0.012	1.7	1.1
Antimycin	0.03	1.1	1.0
Lactic acid	1000	1.3	1.1
Other inducing agents			
Cycloheximide	10	4.8	15.0
Bromodeoxyuridine	10	12.0	8.5

tase DNA product of the Kirsten murine sarcoma virus as a nucleic acid hybridization probe to monitor directly the expression by normal rat cells of those rat sequences incorporated in the Kirsten and Harvey sarcoma virus genomes (MSV-rat sequences). We have also used hybridization to the [^{3}H]DNA product of a representative rat leukemia virus, the Wistar-Furth (WFU), to monitor expression of endogenous leukemia virus RNA (RaLV sequences). For these studies we have used the Fischer rat embryo cell line, where the normal background concentrations of RNA for the endogenous RaLV and for the MSV-rat sequences are both relatively low (*7*).

A previous study in which these methods were used established that rat tumors generally contain levels of both the MSV-rat and RaLV RNA sequences significantly elevated over that seen in most normal tissues, while the concentration of each in cell DNA remains constant (*3*). In this report we characterize these two types of RNA sequences as informational and describe the effect of a variety of agents and conditions on their expression by rat cells in tissue culture.

In view of the absence of known murine sarcoma virus gene products, it was first important to know if the virus genome is composed of informational nucleic acid. We thus examined the MSV-rat and RaLV RNA sequences for tracts of polyadenylate [poly(A)], since the presence of poly(A) on such RNA molecules would suggest that they serve an informational role (*8*). To test indirectly for poly(A) on the MSV-rat and RaLV RNA's, we measured their affinity for oligothymidylate [oligo(T)] cellulose. RNA was isolated from Fischer rat embryo line cells in culture, adsorbed to oligo(T) cellulose, and then the presence of these two RNA species both in the nonadsorbed and in the retained fraction was assayed by molecular hybridization. As described in Fig. 1, 45 percent of the MSV-rat sequences were retained on the column, indicating the presence of associated poly(A). It thus appears that a major fraction of the MSV-rat sequences plays an informational role in normal rat cells. In contrast, only 8 percent of the RaLV RNA sequences were retained on the oligo(T) cellulose, suggesting a much lesser messenger RNA role for these sequences in normal cells. However, it is known that mature type-C leukemia virus RNA does contain poly(A), and in fact is readily translated in vitro (*9*); our result could reflect degradation of this RNA in cells not actually producing virus. These results, combined with the recent observation by Scolnick *et al.* (*7*) that both types of these RNA sequences are apparently associated with polysomes in normal cells, strongly support the notion that both types of RNA are or can be informational.

Table 2. Agents with no effect (less than a twofold alteration) in the concentration of either RNA.

Agent	Concentration (μg/ml)
Pregnandiol	10
Progesterone	10
17β-Estradiol	10
Testosterone	10
Dexamethasone	0.1
Oxandrolone	10
Methandrostenolone	10
Diethylstilbestrol	10
Insulin	10
Diethyl nitrosamine	10
Dimethyl benzanthracene	5
Beryllium sulfate	5
Sodium arsenite	2.5
Dibutyryl cyclic AMP	50
plus theophylline	100
Trypsin (at −24 hours)	0.1 percent*
Trypsin (at −5 hours)	0.1 percent*
Epinephrine	5
Glucose (10×)	10,000
2-Deoxyglucose	500
Galactose	1,000
Thymidine	10
Deoxycytidine	20
Mitomycin C	0.1
Sodium fluoride	25
Sodium arsenate	10
Sodium cyanide	10

*Cells were released from the petri dish in 0.1 percent trypsin at the indicated times and were replated.

Determination of those occasions on which the RaLV and MSV-rat RNA sequences are transcribed by normal cells may offer insight into their possible function. We thus exposed Fischer rat cells in culture to diverse stresses, as described in Table 1, and then monitored the expression of the two types of RNA sequences. Unique among all treatments tested was the response to temporary exposure of Fischer rat cells to anaerobic culture conditions. This treatment had a profound effect on elevating the relative concentration of both the MSV-rat and the endogenous leukemia (RaLV) RNA's, in some cases more than 100-fold. This was apparently not caused by blockage of oxidative phosphorylation or electron transport alone, since exposure of cells to a variety of respiratory poisons such as dinitrophenol, oligomycin, and antimycin all failed to mimic the effect of anaerobic conditions. Lactate, which appears during anaerobic stress (*10*), also showed no inducing activity. It thus appears that something specific to anaerobiosis itself, and not a general respiratory failure, can massively increase the expression by normal cells of both the RaLV and MSV-rat RNA sequences.

Numerous other agents had no significant effect (less than twofold alteration) on the expression of either the MSV-rat or RaLV RNA's (Table 2). Bromodeoxyuridine and cycloheximide increased by 5- to 15-fold the concentrations of both the RaLV and MSV-rat RNA sequences. Both agents induce endogenous type-C viruses by elevating transcription in an apparently nonspecific manner (*11*), and Scolnick *et al.* reported that bromodeoxyuridine increases expression of the MSV-rat sequences (*12*).

Both the MSV-rat and RaLV RNA sequences were expressed completely in the anaerobically treated cells (Fig. 2). The monophasic curves obtained with both hybridization assays indicate that there is no differential expression of subclasses of either of these RNA's.

Three possibilities could account for

the increased specific RNA levels: (i) these RNA species may be transcribed at an elevated rate, (ii) specific RNA stabilization might occur, or (iii) since specific RNA concentrations are computed relative to total cell RNA, other cell RNA's (particularly ribosomal RNA) could be degraded at increased rates. To help discriminate among these possibilities, the effect of the transcriptional inhibitor actinomycin D on the kinetics of induction was examined. As shown in Fig. 3, the concentrations of both the MSV-rat and RaLV RNA sequences began to increase significantly within 3 hours of being placed under anaerobic conditions, and continued to increase for at least 48 hours. The induction of the RaLV RNA sequences did lag somewhat behind that of the MSV-rat sequences. Actinomycin D at a concentration of 3 μg/ml totally blocked the buildup of both types of RNA sequences. Accelerated decay can be ruled out, since total RNA recovery per cell varied less than 30 percent (data not shown). This result indicates that increased rates of synthesis are the dominant cause of the elevated concentrations of these RNA's.

Although still substantial, the overall induction response shown in Fig. 3 is approximately tenfold less than that shown in Fig. 2. In fact, the ultimate extent of the response to anaerobic culture is in part dependent upon cell density, with cultures of higher density showing a reduced response (data not shown). We conducted six separate experiments in which Fischer rat cells were treated anaerobically for 48 hours. The mean induction of MSV-rat RNA was 64-fold, with a range of 9 to 170. The mean induction of RaLV RNA was 78-fold, with a range of 10 to 170.

In evaluating the significance of the anaerobic induction of these two types of RNA sequences it is important to determine whether transcription in general is elevated comparably. The increased specific RNA levels seen cannot merely reflect an increase in all RNA production, since specific RNA concentrations were computed relative to total cellular RNA. According to the method of Schincariol and Joklik (*13*), it can be estimated from the $C_rt_{1/2}$ values (*14*) of 1.5×10^{-1} mole $\times$ sec/liter obtained with Kirsten murine sarcoma virus RNA hybridizing to homologous DNA product, and 5×10^{-2} mole $\times$ sec/liter for WFU rat leukemia virus, that MSV-rat RNA constitutes about 1.0 percent of the RNA from the anaerobically treated cells (Fig. 2), and WFU-RaLV homologous RNA about 0.05 percent of this cell RNA. It is thus likely that at most only a few other RNA species could be expressed at comparably elevated levels.

An important issue yet to be resolved is whether these RNA sequences induced by anaerobic stress function in the same fashion as the lower levels of these same RNA's expressed under normal conditions. If the induced RNA molecules have characteristics indicative of messenger activity, anaerobic stress might offer a useful approach for the induction of complete expression of en-

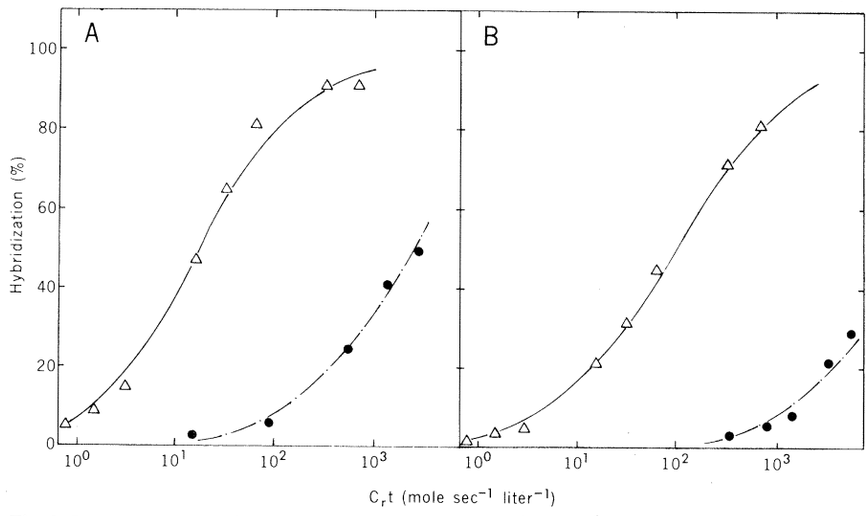

Fig. 2. Increased expression of the MSV-rat and RaLV RNA sequences in anaerobically treated Fischer rat cells. Subconfluent Fischer rat cells in 10-cm plastic petri dishes (Falcon) with 10 ml of tissue culture medium were maintained either in a normal atmosphere plus 5 percent CO_2 or under anaerobic conditions plus 5 percent CO_2 in a GasPak anaerobic chamber (Becton Dickinson) for 48 hours. The latter atmosphere was obtained by charging the GasPak chamber with CO_2 before sealing. The establishment of anaerobic conditions, which results from the catalytic oxidation of hydrogen, was monitored with methylene blue by the method of Smith (*20*). The RNA was extracted and hybridized, as described in Fig. 1, to [^{3}H]DNA prepared from (A) KiMSV (KiMuLV) and (B) WFU-RaLV. Hybridization in (A) is normalized to the rat-specific fraction of this [^{3}H]DNA (40 percent). Symbols: △, hybridization to RNA prepared from Fischer rat cells treated anaerobically; ●, untreated Fischer rat cells.

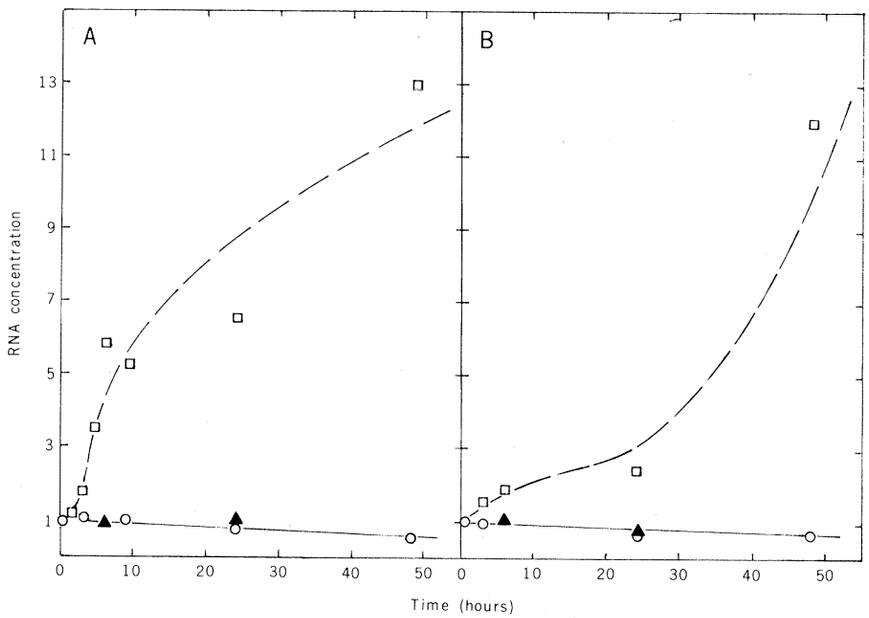

Fig. 3. Kinetics of anaerobic induction of the MSV-rat and RaLV RNA sequences. Fischer rat cells were exposed to anaerobic conditions as described in Fig. 2, except that times were as indicated. The methylene blue test system indicated that approximately 6 hours were required to establish complete anaerobiosis in 10 ml of fluid (but with no cells metabolically active to consume oxygen in the test dishes). RNA was extracted and at least four different amounts were hybridized as described in Fig. 1. RNA concentrations were measured as described in Table 1, and are expressed relative to untreated cells at zero time. Hybridization was to [^{3}H]DNA of (A) KiMSV (KiMuLV) and (B) WFU-RaLV. Symbols: ○, untreated control; □, anaerobic; ▲, anaerobic plus 3 μg of actinomycin D per milliliter.

dogenous leukemia viruses. It might also be expected that mere expression of the two sets of cell genes composing murine sarcoma viruses might result in cellular transformation. However, several criteria show that anaerobically treated cells are not in fact transformed (*15*).

We have established that the sarcoma virus genome is composed (in a recombined form) of two types of normal cellular genetic information; that is, endogenous type-C leukemia virus RNA plus the RNA transcripts of additional specific yet undefined genes. That the resulting sarcoma virus is highly oncogenic compared to its parent leukemia virus suggests that uncontrolled expression of a few cellular genes can directly lead to neoplastic transformation.

Our experiments show that these genes are expressed by normal cells in response to anaerobic stress. These findings are consistent with several possible explanations for their function in the cell. For example, these genes may allow the cells to respond to conditions of curtailed respiration by facilitating fermentation through coding for key enzymes in glycolysis or sugar uptake. This speculation in turn complements Warburg's findings that cancerous tumors in general (*16*), as well as murine sarcoma virus transformed cells (*17*), show increased fermentation, although respiration itself is not suppressed (*18*). Alternatively, induction of endogenous leukemia virus expression may be interrelated with the induction of the MSV-rat sequences, through a more complex series of events.

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Vasoactive Intestinal Polypeptide Occurs in Nerves of the Female Genitourinary Tract

Abstract. *The vasoactive intestinal polypeptide occurs in a richly developed population of nerves that are abundant in the female genitourinary organs. In pigs, cats, rats, and mice these nerves seem to innervate vessels and smooth musculature. Evidence indicates that vasoactive intestinal polypeptide represents a peptide neurotransmitter. Its effects on uterine blood flow and contractility, for example, may be considerable.*

The vasoactive intestinal polypeptide (VIP) isolated by Said and Mutt (*1*) represents a new neuronal peptide (*2–4*). Our immunocytochemical studies have demonstrated that VIP nerves are not confined to the gut but occur also in the brain (*3*) and around cranial blood vessels (*4*). Here we report that nerves containing this vasodilatory peptide can be found intimately associated with vessels and smooth muscle of the female genitourinary tracts of pigs, cats, rats, and mice.

Pigs were obtained at a nearby abattoir, cats were killed with Nembutal, and rats and mice were decapitated. Feline and murine tissue was used for immunocytochemistry and feline and porcine material for radioimmunoanalysis. Samples were taken from ovaries, Fallopian tubes, uterus, vagina, kidneys, ureters, urinary bladder, and urethra. For immunocytochemistry, samples were frozen in melting Freon-22 and freeze-dried, and were then vapor-fixed with diethylpyrocarbonate and embedded in paraffin as described previously (*3*, *4*). Sections (3 μm thick) were cut and the paraffin was removed. Antiserum to VIP [No. 5603-6, see (*5*)] was allowed to react with the sections, and the site of antigen-antibody reaction was revealed by means of the unlabeled peroxidase (PAP) method of Sternberger (*6*). The controls used were those recommended by Sternberger (*6*) and included the application of antigen-inactivated antiserum (containing 30 nmole of highly purified porcine VIP per milliliter of antiserum diluted 1:80).

Specimens for radioimmunoanalysis

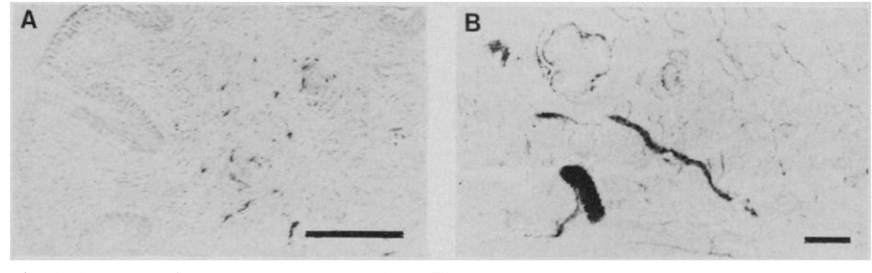

Fig. 1. (A) A section from cat endometrium. The black-stained VIP-containing nerves occur scattered between the glands; some of these nerves are visible around a small artery. (B) A section from cat uterine wall. VIP-containing nerves occur either singly or in large bundles. In the upper left corner three myometrial arterial branches are surrounded by VIP-containing nerves in an innervation-like pattern. The sections were stained with the immunoperoxidase procedure for demonstrating VIP (*6*), and photographs were taken in a Nomarski-type interference contrast microscope to reveal histological details. (Scale bars, 100 μm)