growth by TPA do not carry out TPA-induced DNA synthesis at G_0 (*23*).

The effective concentrations of TPA and DHTB for inhibition of epidermal growth factor binding and enhancement of 2-deoxyglucose uptake and the effective concentrations of TPA for enhancement of transformation, stimulation of DNA synthesis in arrested cells, and inhibition of [^{3}H]phorbol-12,13-dibutyrate binding agree with data for other cell lines (*11, 12*), indicating that the susceptibility of A31-1-1 cells to the effects of DHTB and TPA is similar to that of other cell lines. We recently found that DHTB and TPA, applied by painting, enhance the development of skin tumors initiated by 7,12-dimethylbenz[*a*]anthracene equally well (*11*). However, the results shown here suggest that DHTB may be more potent than TPA in promoting tumor development in vivo under certain circumstances.

The extremely low effective dose of DHTB for the enhancement of transformation (less than 10^{-10} g/ml) indicates that environmental promoting agents like teleocidin, the parental compound of DHTB, strongly influence the development of human cancer and that the effects of DHTB reflect interference with the control of cell function by hormone-like substances. Further studies with DHTB and teleocidin are necessary to elucidate these issues.

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References and Notes

1. T. J. Slaga, A. Sivak, R. K. Boutwell, Eds., *Carcinogenesis* (Raven, New York, 1978), vol. 2.
2. I. Berenblum, in *Cancer*, F. F. Becker, Ed. (Plenum, New York, 1975), vol. 1, pp. 323–344.
3. E. Hecker, in *Carcinogenesis: Fundamental Mechanisms and Environmental Effects*, B. Pullman, P. O. P. Ts'o, H. Gelboin, Eds. (Reidel, Dordrecht, Netherlands, 1980), pp. 257–272.
4. M. Takashima and H. Sasaki, *Bull. Agric. Chem. Soc. Jpn.* **24**, 647 (1960).
5. ———, K. Arima, *Agric. Biol. Chem.* **26**, 660 (1962).
6. T. Kakunaga, *Int. J. Cancer* **12**, 463 (1973).
7. ——— and J. D. Crow, *Science* **209**, 505 (1980).
8. S. Mondal, D. W. Brankow, C. Heidelberger, *Cancer Res.* **36**, 2254 (1976).
9. A. R. Kennedy, S. Mondal, C. Heidelberger, J. B. Little, *ibid.* **38**, 439 (1978).
10. H. Fujiki, M. Mori, M. Nakayasu, M. Terada, T. Sugimura, *Biochem. Biophys. Res. Commun.* **90**, 976 (1979).
11. ———, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3872 (1981).
12. K. Umezawa, I. B. Weinstein, A. Horowitz, H. Fujiki, T. Matsushima, T. Sugimura, *Nature (London)* **290**, 411 (1981).
13. H. Hoshino, M. Miwa, H. Fujiki, T. Sugimura, *Biochem. Biophys. Res. Commun.* **95**, 842 (1980).
14. I. B. Weinstein, H. Yamasaki, M. Wigler, L. S. Lee, P. B. Fisher, A. M. Jeffery, D. Grunberger, in *Carcinogens*, A. C. Griffin and C. R. Shaw, Eds. (Raven, New York, 1979), pp. 399–418.
15. H. Yamasaki, in *Molecular Cellular Aspects of Carcinogen Screening*, R. Montesano, L. Tomatis, H. Bartsch, Eds. (International Agency for Research on Cancer, Lyon, France, 1980), pp. 91–111.
16. G. C. Mueller, P. W. Wertz, C. H. Kwong, K. Anderson, S. A. Wrighton, in *Carcinogenesis: Fundamental Mechanisms and Environmental Effects*, B. Pullman, P. O. P. Ts'o, H. Gelboin, Eds. (Reidel, Dordrecht, 1980), pp. 319–333.
17. P. Dicker and E. Rozengurt, *Nature (London)* **276**, 723 (1978).
18. L. S. Lee and I. B. Weinstein, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5168 (1979).
19. R. K. Boutwell, *CRC Crit. Rev. Toxicol.* **2**, 419 (1974).
20. P. E. Driedger and P. M. Blumberg, *Cancer Res.* **37**, 3257 (1977).
21. A. R. Peterson, S. Mondal, D. W. Brankow, W. Thon, C. Heidelberger, *ibid.*, p. 3223.
22. I. B. Weinstein, M. Wigler, C. Pietropaolo, in *Origins of Human Cancer*, H. H. Hiatt, J. D. Watson, J. A. Winsten, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1977), pp. 751–772.
23. N. H. Colburn, E. J. Wendel, G. Abruzzo, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
24. We thank R. S. Day and M. Mattern for their critical reading of the manuscript.
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The Molecules That Initiate Cardiac Hypertrophy Are Not Species-Specific

Abstract. *Extracts from hypertrophying dog hearts perfused through isolated rat hearts increase the synthesis of messenger RNA and initiate hypertrophy in the treated hearts. Total RNA extracted from experimental and control hearts was translated in vitro and hybridized with polyuridylate. Synthesis of protein and polyadenylate-containing RNA was greater in rat hearts perfused with extracts of hypertrophying dog hearts than in control hearts. The results demonstrate that molecules from hypertrophying dog hearts are not species-specific since they are effective in stimulating transcription of messenger RNA in rat hearts as well as in dog hearts.*

The canine heart, when provoked to hypertrophy by an increased work load, synthesizes water-soluble, extractable molecules that can initiate hypertrophy (as defined by increased protein synthesis) when perfused through isolated, beating (but nonworking) canine hearts (*1*). The possibility that cells generate growth-regulating molecules in response to stress suggests that these molecules may be fundamental to cell function and may exhibit little or no species specificity. If so, extracts derived from the hearts of one species should induce hypertrophy in the hearts of other species. Accordingly, we modified the protocol that we had used for the isolated dog hearts to determine whether canine cardiac extracts would induce responses in rat hearts similar to those in dog hearts.

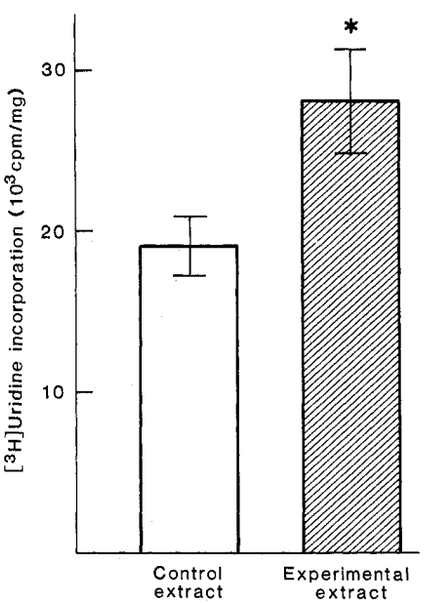

Fig. 1. Incorporation of [^{3}H]uridine into newly synthesized RNA in rat hearts during perfusion. Rat hearts were perfused for 1 hour each with Krebs solution plus normal canine cardiac extract (control extract) and Krebs solution plus hypertrophying canine cardiac extract (experimental extract). Isotope incorporation was 48.7 percent higher in the experimental extract than in the control extract ($t = 2.45$, d.f. = 8, $^*P < .05$). Five hearts were used in each group.

Left ventricular hypertrophy was initiated in dogs by banding the ascending aorta. Six hours later, the heart was extirpated, and the left ventricle was finely chopped. The tissue was frozen with liquid nitrogen and stored at −80°C. Normal canine hearts for control purposes were prepared in identical fashion. On the day of perfusion experiments, 5 to 6 g each of hypertrophying and normal tissue was homogenized in distilled water at a ratio of 1:2 (weight to volume) with a Polytron (Brinkmann) for approximately 15 seconds at the highest speed. The homogenate was centrifuged at 17,000*g* for 30 minutes at 4°C. The supernatant was decanted and placed on ice until used.

Hearts were excised from male, 12-month-old, albino rats. The aortas were cannulated and placed in iced saline. The left ventricle was vented by ventriculot-

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Table 1. Relative amount of poly(A) in various RNA preparations, measured as amount of radioactivity in acid-insoluble materials after hybridization with [^{3}H]poly(U) and digestion with ribonuclease. Values for radioactivity are means ± S.E.M. Group 1 was significantly different from group 2 ($P < .05$) and group 3 ($P < .01$). Groups 2 and 3 were not significantly different.

Group	Treatment	No. of hearts	No. of repetitions	Radioactivity (count/min)
1	Experimental extract	4	9	2829 ± 86
2	Control extract	4	9	2409 ± 168
3	Krebs control	4	9	2408 ± 89

omy to prevent the heart from generating pressure. Each heart was then connected to a separate perfusion apparatus that consisted of a constant-pressure reservoir and collector that maintained a temperature of 37°C and a pressure of 100 mmHg.

Experiments were done in three groups. In group 1, the perfusate consisted of oxygenated Krebs solution to which hypertrophying cardiac extract (2 ml per heart) had been added. In group 2, a control group, the perfusate consisted of Krebs solution to which 2 ml of normal cardiac extract had been added. In group 3, which served as a control for both groups 1 and 2, the perfusate consisted of Krebs solution only. The hearts were perfused for 1 hour, and viability was demonstrated by continued contractions. The hearts perfused were frozen in liquid nitrogen and stored at −80°C.

For direct labeling experiments, a total of five hearts was used for each group. In these experiments, 300 μCi of [^{3}H]uridine was included in each of the perfusates. After 1 hour of perfusion, the hearts were homogenized in 10 ml of 6*M* guanidine hydrochloride and centrifuged at 17,000*g* for 30 minutes at 4°C. Portions of the supernatant were used for the determination of [^{3}H]uridine incorporation and protein content; for each determination, 100 μl of the supernatant was diluted with 1.4 ml of water, and 1.0 ml of 25 percent trichloroacetic acid (TCA) was added. The precipitate was then collected on a GF/A filter, washed with 8 percent TCA, dried, and counted in 10 ml of Aquasol in a scintillation counter (Beckman). Protein content was determined (*2*) with bovine serum albumin used as a standard.

The direct labeling experiments revealed a substantial increase in RNA synthesis in those rat hearts perfused with extracts from canine hypertrophying hearts as compared to those hearts perfused with control extracts (Fig. 1). Corresponding increases in protein synthesis were observed when [^{35}S]methionine was used to label newly synthesized proteins, but results were more variable than those for RNA synthesis (not shown).

Our most commonly used procedure for determining whether hypertrophy had been initiated was to measure the translational activity of total cytoplasmic RNA extracted from the treated hearts. This procedure was cheaper than the direct labeling techniques and was also more convenient for repeated laboratory use. Total RNA was isolated from perfused hearts (*3*). The quantity of RNA was estimated by the absorbance at 260 nm, one absorbance unit of RNA being equivalent to 50 μg (*4*). The purity of RNA was determined by the absorption spectrum which showed a symmetrical peak at 259 nm; the ratio of absorbance at 260 nm to that at 280 nm was approximately 2:1. The translational activity of the RNA was assayed with a commercially available [^{35}S]methionine translation kit (New England Nuclear) (*5*). The quality of RNA was assessed by electrophoresis and autoradiography of the translation products. The autoradiograms showed multiple clear bands of synthesized proteins ranging in molecular weight from < 12,400 to > 69,000.

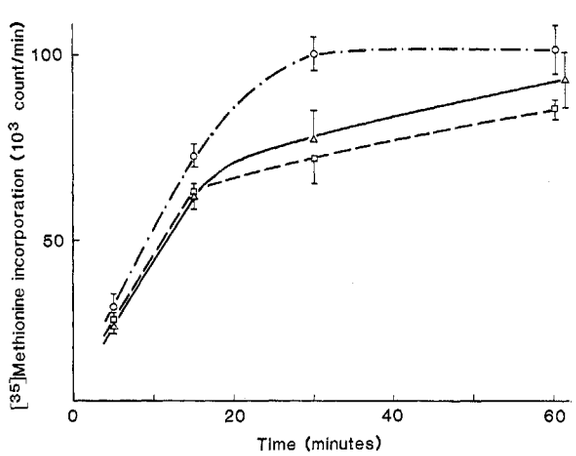

Fig. 2. Incorporation of [^{35}S]-methionine into translation products directed by cytoplasmic RNA extracted from rat hearts each perfused for 1 hour with (——) Krebs solution alone, (– – –) Krebs solution plus normal canine cardiac extract, or (·—·—) Krebs solution plus extracts from hypertrophying canine hearts. Five hearts were used in each group, and each measurement was made in triplicate. Values for group 1 were significantly greater than the combined values for groups 2 and 3 ($P < .05$).

Samples of RNA from 15 perfused hearts, grouped as above, generated data points for each group at four time points. Each point represents the mean ± standard error of the mean (S.E.M.) of five hearts. Hearts in each group were tested on the same day with the same translation kit, but, as each experiment was repeated three times, three translation kits were used in total for this part of the experiment. The three curves generated by the 60 data points were analyzed by two-factor analysis of variance. The hearts perfused with hypertrophying cardiac extract (group 1) have significantly greater translational activity than that from groups 2 and 3 combined (Fig. 2). No significant difference was found between groups 2 and 3.

To determine whether changes in translational activity of the RNA from isolated perfused hearts represented a change in the rate of translation of preexisting messenger RNA (mRNA) (translational control) or a relative increase in the amount of mRNA in relation to total RNA (transcriptional control), we determined the relative quantity of polyadenylated [poly(A)] RNA in the preparations by hybridizing the RNA with ^{3}H-labeled polyuridylate [poly(U)] (*6*). In a total volume of 2 ml, each assay mixture contained 100 μCi of [^{3}H]poly(U) ([5,6-^{3}H]poly(U)) with a specific radioactivity of 5 Ci per millimole of uridine monophosphate and 10 μg of RNA in a buffer consisting of 0.01*M* tris-HCl (*p*H 7.5), 0.2*M* NaCl, and 0.01*M* $MgCl_2$. The RNA in the mixture was allowed to hybridize at 25°C for 30 minutes. Unhybridized RNA was digested at 37°C for 15 minutes with 40 μg of pancreatic ribonuclease A (Sigma). After ribonuclease digestion, 50 μg of carrier DNA and 0.5 ml of 20 percent TCA were added, and the samples were cooled at 0°C for 10 minutes. Precipitated materials were collected on GF/A filters, washed with ice-cold 1 percent TCA, dried, and counted in 10 ml of Aquasol (*7*).

The poly(A) hybridization data were obtained from 12 isolated perfused hearts. The mean ± S.E.M. was calculated from nine replicates in four independent experiments, each consisting of

three groups as above. The significance of the difference between the groups was calculated by Student's *t*-test. These data demonstrated a clear increase in the amount of poly(A) mRNA in relation to total RNA in the hearts perfused with hypertrophying extract, in comparison with hearts perfused with control extract or hearts perfused with Krebs solution only. There is no significant difference between the latter two groups (Table 1).

Although our assay system, involving treatment of intact beating hearts, is cumbersome, it provides clear repeatable results. We have tried simpler systems based on tissue slices, but the results were erratic. In tissue slices, cell contact arrangements were distorted, heart cells were not contracting, the vascular endothelium was not intact, and molecules were not bound or metabolized by the endothelium before the molecules could enter parenchymal cells (*8*). We believe that essentially normal anatomic and physiologic arrangements of an organ are required in order to obtain reliable biological responses. Such intact organ preparations are imperative when attempting to demonstrate the presence or absence of growth-initiating factors derived from tissue extracts.

The discrete early spread of translation curves that plateau at approximately 30 minutes suggests that increased amounts of mRNA account for the increased translation. An alternative, refuted by our experiments, is an increase in translational activity of preexisting mRNA. An increase in mRNA is substantiated by the increased proportion of poly(A) RNA, as determined by poly(U) hybridization, in the experimental groups. The molecules that provoke hypertrophy are probably basic to cell metabolism and appear to have been strongly conserved during evolution. Our results indicate that the controlling molecules exert their cellular effects by increasing transcription; that is, by activating genes.

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References and Notes

1. G. L. Hammond, E. Weiben, C. L. Markert, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2455 (1979).
2. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
3. M. J. Ernest and P. Feigelson, *ibid.* **253**, 319 (1978). Heart tissue (average, 1.7 g) was homogenized with a Polytron for 15 seconds at the highest speed in 25 ml of 0.02*M* sodium acetate (*p*H 5.2) containing 6*M* guanidine hydrochloride and 0.01*M* dithiothreitol. The homogenate was centrifuged at 20,000*g* for 30 minutes at 4°C, and the supernatant was layered over a 12-ml cushion of 5.7*M* CsCl and 0.1*M* EDTA in a 40-ml centrifuge tube. The samples were centrifuged at 110,000*g* for 16 hours at 20°C (Beckman L8-70 centrifuge with an SW-28 rotor). After centrifugation, the RNA pellet was dissolved in 0.6 ml of water, adjusted to 2 percent potassium acetate (*p*H 5.2), and precipitated with two volumes of ethanol at −20°C for 3 hours; the precipitate was collected by centrifugation at 17,300*g* for 10 minutes. This process was repeated three times. After final centrifugation, the RNA pellet was washed twice with 80 percent ethanol, dried under a stream of N_2, dissolved in a minimal volume of water, and stored at −80°C.
4. R. D. Palmiter, R. Palacios, R. T. Schimke, *J. Biol. Chem.* **247**, 3296 (1972).
5. The 5-μl translation product was diluted in 1 ml of water and decolored with 0.5 ml of 0.1*N* NaOH containing 0.5*M* H_2O_2 and 10 m*M* methionine. After 15 minutes, 1.0 ml of 25 percent TCA was added. The precipitate was collected on a GF/A filter, washed with 8 percent TCA, and counted in 10 ml of Aquasol in a scintillation counter (Beckman LS 7000). The procedure described by the manufacturer was followed except that the final volume for each assay was doubled to 50 μl. Each assay mixture contained 5 μg of RNA, 100 μCi of [^{35}S]methionine, 0.08*M* potassium acetate, 650 μ*M* $MgCl_2$, and 11 μl of translation cocktail consisting of Hepes buffer and energy sources. The mixture was incubated at 30°C. At 5, 15, 30, and 60 minutes, 5-μl portions were withdrawn from the samples and processed for measurement of radioactivity in acid-insoluble materials.
6. W. R. Jeffrey and G. Brewerman, *Biochemistry* **13**, 4633 (1974).
7. Background radiation, as determined from blanks (assays without added RNA), was usually found to be about 100 to 150 count/min. This was subtracted from samples before comparisons were made.
8. G. L. Hammond, L. H. Cronau, D. Whittaker, C. N. Gillis, *Surgery* **81**, 716 (1977).
9. Supported by NIH grant HL 25699-01 and a grant from the Fannie E. Ripple Foundation.

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Relaxation of Isolated Gastric Smooth Muscle Cells by Vasoactive Intestinal Peptide

Abstract. *Vasoactive intestinal peptide caused a prompt, dose-dependent relaxation of isolated gastric smooth muscle cells of the guinea pig and a significant increase in intracellular adenosine 3′,5′-monophosphate coincidentally with optimum relaxation. Relaxation was augmented by a threshold concentration of isobutyl methylxanthine. The direct relaxant effect of vasoactive intestinal peptide and the distribution of nerves containing this peptide to circular smooth muscle support the view that vasoactive intestinal peptide is the neuromuscular transmitter of enteric inhibitory nerves.*

Neurons that show positive reactions with antibody to vasoactive intestinal peptide (VIP) are present in the myenteric and submucosal plexuses of the gut; their axons innervate all cell types including other neurons, smooth muscle cells, and mucosal cells (*1*, *2*). Axons arising from VIP neurons in the myenteric plexus project in a caudal direction forming varicose rings around VIP-reactive and nonreactive neurons. Other axons project into the circular muscle layer and run parallel with smooth muscle cells. The topography of VIP neurons and nerve fibers and the known relaxant effect of VIP on circular smooth muscle (*3*–*5*) are consistent with its being the transmitter of the enteric inhibitory nerves. These nerves mediate a series of descending inhibitory reflexes which facilitate the passage of material traveling down the digestive tract (*4*).

Evidence favoring VIP as the principal transmitter of this pathway includes release of VIP into the venous effluent concomitantly with neurally induced gastric relaxation (*6*) and partial blockade of neurally induced relaxation of the lower esophageal sphincter by VIP antiserum (*7*). The design of the experiments that gave these results left open the possibility that VIP was chiefly an interneuronal transmitter. In more recent studies, Angel *et al.* (*8*) showed that neurally induced relaxation of the gastric muscularis mucosae and concomitant VIP release could be abolished by the axonal blocking agent tetrodotoxin; relaxation was also abolished by antiserum to VIP.

Decisive evidence that VIP acts directly on smooth muscle cells rather than indirectly by way of local neural pathways would be demonstration of an effect of VIP on isolated smooth muscle cells devoid of neural elements. We have developed a procedure for isolation of smooth muscle cells from the stomach of several mammalian species, including humans, and devised an image-splitting technique for measurement of contraction and relaxation (*9*). In the present study, we demonstrate the presence of high-affinity VIP receptors that are capable of mediating prompt relaxation of isolated gastric smooth muscle cells of the guinea pig; this relaxation occurs concomitantly with an increase in intracellular levels of adenosine 3′,5′-monophosphate (cyclic AMP).

Cells were isolated from the stomach of guinea pigs as described (*9*) (see legend to Fig. 1). Isolated cells are in a state of optimum relaxation; therefore, the

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