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CONTROL OF *IN VIVO* PROTEIN SYNTHESIS AND PEROXIDASE FORMATION BY DNA-CONTAINING EXTRACTS OF NORMAL AND CROWN GALL TISSUES*

JOSEF F. NOVAK† and ARTHUR W. GALSTON

Department of Biology, Yale University, New Haven, CT 06520, U.S.A.

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; bean; hypocotyl; crown gall; protein synthesis; peroxidase; DNA.

Abstract—Nucleic acids extracted from normal bean hypocotyl tissue (NE) and crown gall tumors (TE) affect amino acid incorporation into protein and the development of peroxidase activity when vacuum infiltrated into normal receptor tissues. TE enhances and NE inhibits both processes; NE from successively older tissues produces progressively greater inhibitions per unit of infiltrated nucleic acid. The active material has an absorption maximum at 257 nm with an $A_{260:280}$ ratio of more than 2·0. On acrylamide gel electrophoresis it shows a small DNA peak, four typical *r*-RNA peaks and a small low molecular weight RNA peak. Activity in such extracts is completely destroyed by hydrolysis with 0·3 N KOH or DNAase; RNAase is only slightly effective and pronase ineffective. It is deduced that the effective material contains DNA that may be complexed with RNA or other materials in the extract. Pretreatment of donor tissues with actinomycin D or 5-fluorouracil diminishes or annuls the activity of the extract. Pretreatment of receptor tissue with actinomycin D inhibits the action of TE but not of NE; pretreatment with cycloheximide prevents the action of both NE and TE.

INTRODUCTION

We have previously reported that RNA extracted from tobacco pith cells and vacuum infiltrated into similar receptor cells alters patterns of isoperoxidase development in the receptor tissue [1,2]. Since a high titer of such activity in RNA extractable from donor pith cells is dependent on a high auxin supply, we inferred that the previously observed auxin-induced repression of certain isoperoxidases [3] is mediated through control of the synthesis of particular RNA molecules, possibly mRNA. Similar hormone-like effects of RNA extracts derived from hormone-treated animal tissues have also been frequently observed (see Ref. 4, and references therein). In the present related study, we have found that nucleic acid preparations from normal and crown gall tissues of bean hypocotyl, prepared as in our earlier studies, can differentially control the rates of protein synthesis and peroxidase activity in appropriate receptors. Contrary to our previous experience, the biological activity appears to reside in DNAase-sensitive, rather than in RNAase-sensitive material. These effects seem to offer possible insights into events controlling tumorigenesis, ageing and senescence.

RESULTS

Analysis of normal and crown gall tissue at various positions on bean hypocotyl revealed that tumor cells contain much more protein per unit fr. wt and much higher peroxidase activity per unit protein than do normal or untransformed cells (Table 1). It also appears that the normal tissues

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† Present address: Department of Biology, Princeton University, Princeton, New Jersey, U.S.A.

Table 1. Total protein and peroxidase specific activity in control and tumor hypocotyl tissues of 37-day-old plants inoculated on day 5 with *Agrobacterium tumefaciens* strain T37

Tissue	Segment of hypocotyl	Sol. prot. (mg/g fr. wt)	Units peroxidase activity per mg protein
Control (uninoculated)	u*	1·890	30·3
	m	1·547	33·9
	l	1·538	34·8
Crown gall tumor	u	5·125	122·1
	m	6·636	94·7
	l	4·682	52·5
Normal tissue opposite the tumor	u	3·137	32·1
	m	1·947	33·7
	l	2·432	36·5

* u— upper; m—middle; l—lower.

opposite the tumor show increased levels of protein in advance of obvious tumorization. This led to a search for a tumor-derived factor which might increase protein synthesis in normal receptor cells. Nucleic acid extracts from tumors (TE) had such an effect, while similar extracts from normal cells (NE) acted in the opposite direction.

Figure 1 shows that when normal tissue was used as receptor, NE decreased the efficiency of incorporation (IE) of [^{14}C]-leucine into TCA-insoluble material at all concentrations employed, while TE increased IE up to about 0·7 mg/ml of donor extract (expressed as RNA). NE from progressively older donor tissues produced greater and greater inhibition of amino acid incorporation (Fig. 2) as shown by the lowered specific activity of the extracted proteins. The response was also influenced by the age of the normal receptors, in terms of both peroxidase activity and absolute amounts of radioactivity incorporated into proteins (Table 2). Calculated incorporation efficiency values from the data in Table 2 confirm the conclusion that aged normal receptors retain considerable capacity to respond to the exogenous nucleic acids (Fig. 3).

Both the promotive activity of TE and the inhibitory activity of NE on incorporation efficiency in normal receptors were destroyed by hydrolysis of the extracts with 0·3 N KOH. Activity was also diminished by exposure to specific enzymes: deoxyribonuclease was most effective, ribonuclease had slight activity and pronase was essentially inactive

Table 2. The effect of the age of receptor tissues on the magnitude of the response to exogenous nucleic acid

Donor	Receptor	Peroxidase U/g fr. wt		^{14}C Activity (cpm/g fr. wt) Insoluble		Soluble		Total		Peroxidase U/mg protein	
		$\times 10^{-3}$	(%)	$\times 10^{-3}$	(%)	$\times 10^{-3}$	(%)	$\times 10^{-3}$	(%)	$\times 10^{-3}$	(%)
Buffer	N_3	30·2	(100)	523	(100)	530	(100)	1053	(100)	5788	(100)
NE	N_3	21·0	(69)	476	(90)	743	(140)	1219	(155)	4411	(76)
TE	N_3	31·5	(104)	596	(114)	594	(113)	1190	(113)	5553	(96)
Buffer	N_5	15·6	(100)	217	(100)	275	(100)	492	(100)	3735	(100)
NE	N_5	13·8	(88)	189	(86)	301	(110)	490	(99)	3643	(97)
TE	N_8	18·4	(118)	228	(105)	298	(108)	626	(107)	4549	(122)
Buffer	N_8	7·4	(100)	14	(100)	197	(100)	201	(100)	3144	(100)
NE	N_8	5·3	(72)	13	(94)	294	(157)	307	(152)	1789	(57)
TE	N_8	11·9	(162)	49	(348)	340	(182)	390	(193)	3606	(115)

NE was obtained from 12-day-old hypocotyls and TE from 80-day-old tumors. Normal receptor tissues N_3, N_5 and N_8 were prepared from segments of hypocotyls aged $3\frac{1}{2}$, $5\frac{1}{2}$, and $8\frac{1}{2}$ days. After vacuum infiltration with donor extracts, receptors were post-incubated for 12 hr in Heller's medium containing a mixture of [^{14}C]-amino acids (New England Nuclear). The final specific activity of the medium was 1 μCi/ml. Each number in the table represents an average of two or three independent assays.

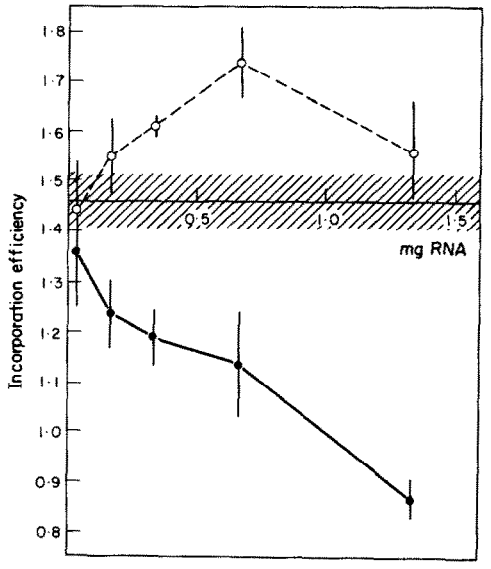

Fig. 1. Response of normal receptor tissue to various levels of infiltrated NE and TE, expressed in terms of incorporation efficiency. ——NE; – – – –TE.

(Fig. 4). Acrylamide gel electropherograms confirmed the breakdown of the indicated nucleic acids by the specific nucleases (Fig. 5).

Pretreatment of normal donor tissues with actinomycin D (AMD) or 5-fluorouracil (5-FU) resulted in the production of inactive or less active preparations (Table 3). Peroxidase activity and the

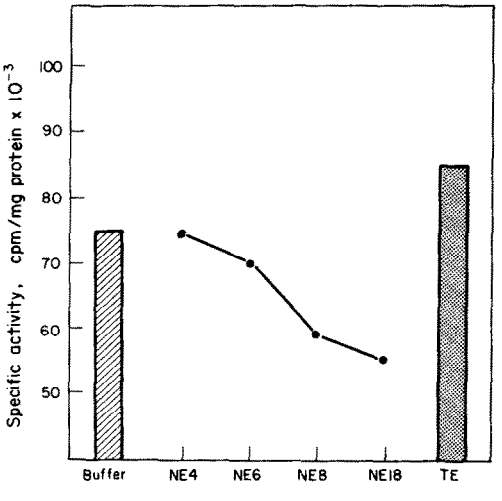

Fig. 2. Specific activity of the protein fraction of normal receptors infiltrated with buffer, TE and NE from tissue of various ages.

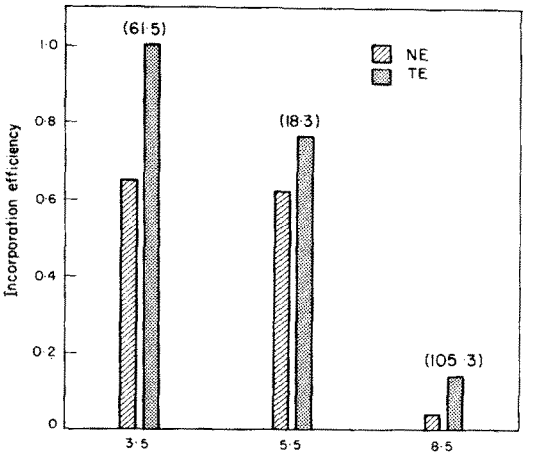

Fig. 3. Differences in incorporation efficiency induced by standard NE and TE on normal receptor tissues of different ages. The percentage difference figures (in parenthesis) were calculated according to the formula: [TE − NE)/(NE + TE/2] × 100.

capacity for protein synthesis were also measured in such tissues. Provided that the measurements are done immediately after the AMD treatment,

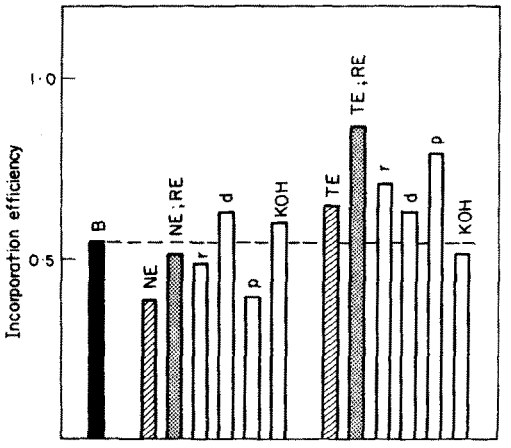

Fig. 4. The effect of KOH and various enzymes on the promotive action of TE and inhibitory action of NE on incorporation efficiency in normal receptors. All enzymatic digestions were performed at 37° for 1 hr. B = buffer; RE = reextracted; r = ribonuclease (Worthington Biochem. Corp.; pancreatic; assay was performed in 0·1 M sodium acetate buffer, pH 5·0 containing 1 mg of RNA/ml and 10 μg/ml of the enzyme); d = deoxyribonuclease (RNAase free; Worthington Biochem. Corp., assayed in the same buffer as above but enriched with 0·05 M $MgSO_4$; 20 μg of enzyme per 1 mg of RNA and 1 ml of solution); p = pronase (Calbiochem. Comp., 1 hr self digested at 30°; assay mixture consisted of 0·2 M Tris–HCl buffer, pH 8·5, 1 mg/ml RNA, and 60 μg/ml of the enzyme).

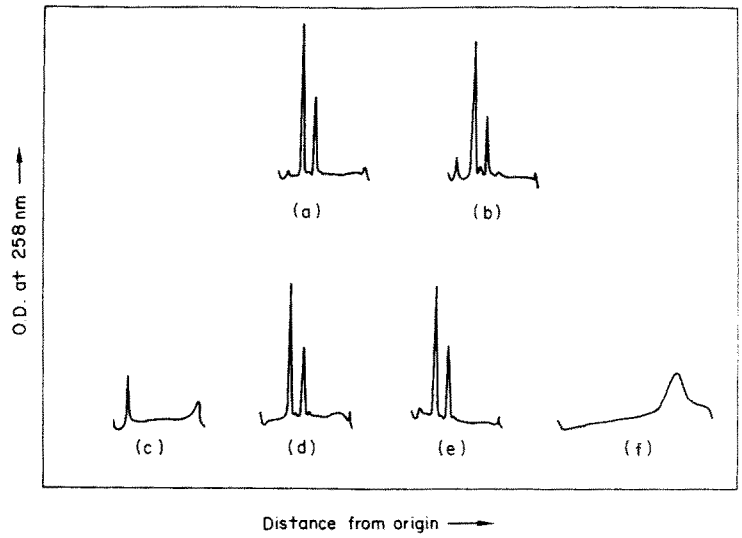

Fig. 5. Acrylamide gel electrophoresis of extracts tested for biological activity on normal and tumorous tissues. Acrylamide gel patterns of NE (a) and TE (b) were treated with enzymes (see legend to Fig. 4) and KOH. The samples were reextracted for purpose of biological assay. The digestion of both NE and TE gave similar patterns: only TE patterns are shown: (c) ribonuclease, (d) deoxyribonuclease, (e) pronase, (f) 0·3 N KOH: treatments lasted 1 hr at 37°, treated samples were dialysed and phenol-reextracted before both electrophoresis and biological assay).

both rate of protein synthesis and peroxidase activity were higher than in the untreated controls. This may imply the usual production, in normal tissues, of macromolecules restraining the rate of overall protein synthesis; similar effects have been noted previously for the plant peroxidase complex [5] and a number of animal systems [6,7]. If however, the rate of protein synthesis or peroxidase activity is measured several hours after the AMD incubation period has ended, the "superinduction effect" of the drug disappears (Table 4). Under such conditions the normally promotive effect of subsequently administered TE is almost completely annulled, but the inhibitory action of NE is unaffected. Similar prior incubation with cycloheximide prevents substantial synthesis of all proteins, including peroxidase. Additional treatment of such cycloheximide pretreated tissues with NE or TE does not alter the capacity of the tissue to change its peroxidase activity. The data indicate that

Table 3. Effect of pretreatment of normal donor with actinomycin D or 5-fluorouracil on activity of extracted NE. Development of peroxidase activity in normal receptors used to measure the effect

Infiltration	Peroxidase activity U/g fr. wt ($\times 10^{-3}$)	Relative	Peroxidase sp. act. U/μg protein	Relative
Buffer	70·5	100	21·0	100
NE	63·0	89	17·0	81
AMD:NE	75·5	105	26·8	127
5 FU:NE	66·1	94	18·6	89
TE	84·9	120	23·0	109

Sterile 2½-day-old seedlings in petri dishes transferred to buffer ± AMD (4 μg/ml) or 5-FU (0·1 mM) for an additional 12 hr. Peroxidase activity determined with guaiacol. Original tissue 29·6 U/g fr. wt ($\times 10^{-3}$) and 14·2 U/μg protein: excised, incubated (uninfiltrated) tissue 49·0 U/g fr. wt ($\times 10^{-3}$) and 14·2 U/μg protein. Approx 500 μg tissue infiltrated with 1·5 ml 10 mM Tris-HCl, pH 7·4 ± 500 μg nucleic acid for 25 min; postincubation 12 hr.

Table 4. Effect of preincubation of normal receptor in actinomycin D or cycloheximide on subsequent effect of NE and TE on developed peroxidase activity

Pretreatment	Infiltration	Peroxidase activity U/g fr. wt ($\times 10^{-3}$)	Relative	Peroxidase sp. act. U/μg protein	Relative
Distilled water	Buffer	323	100	77·5	100
	NE	206	64	63·2	82
	TE	495	153	92·1	119
AMD (5 μg ml)	Buffer	117	100	24·8	100
	NE	78·6	67	20·5	83
	TE	122	104	29·8	120
CH (5 μg/ml)	Buffer	83·8	100	15·8	100
	NE	67·2	80	14·3	91
	TE	60·3	72	12·1	77

Initial tissue has a peroxidase activity of 134 U/g fr. wt ($\times 10^{-3}$) and a peroxidase specific activity of 19·8 U/μg protein. Buffer was 10 mM Tris–HCl, pH 7·5 + 3% sucrose. Receptor tissues (1–2 mm sections prepared from 3·5-day-old sterile bean hypocotyls) were first given a 3 hr long treatment with or without (control) the inhibitors, after which the tissues were shaken in drug-free media for a 15 hr period. This was followed by 25 min long period of infiltration with NE or TE and finally a 12-hr postincubation period in dark.

translation is probably required for the effects of NE and TE to be manifested. Experiments with amino acid incorporation yielded similar results.

DISCUSSION

The effective entry of exogenous DNA and RNA into intact cells and protoplasts of various eukaryotic cells has been suggested by the work of many investigators [8,9]. Several recent achievements [10–14] have especially strengthened the hopes for regulated uptake and expression of exogenous DNA molecules in cells in plant origin. This report simply extends the list of apparently successful efforts. What is striking about our results is their possible implications for tumorigenesis, ageing and senescence.

Why does a crown gall tumor on the bean hypocotyl grow so much more rapidly than the surrounding normal tissue? One possible explanation, a greater rate of protein synthesis, is suggested by our studies showing an increased rate of incorporation of labeled amino acids into TCA-insoluble material following administration of TE to normal receptors. TE may or may not contain the tumor-inducing principle [15,16]; in any event, it apparently carries some DNA sequence capable of producing enhanced protein synthesis in normal cells. Protein synthesis in tumor cells is not further promoted by additional doses of this material, indicating that they have an optimal content of the active material.

The activity of the NE from progressively older normal donors increased steadily (Fig. 2). The calculated difference in incorporation efficiency induced by NE and TE, respectively, was surprisingly highest in the oldest normal tissue (Table 2 and Fig. 3). Both observations might be explained by phenomena often associated with ageing, i.e. by a progressively more repressed genome in older cells [17] as well as by the build-up of specific inhibitory substances during differentiation [18,19].

The DNA nature of the active material is indicated by its sensitivity to DNAase. Since activity is also destroyed by treatment with alkali, the possibility is suggested that the active DNA is double-stranded or that it is associated with some alkali-labile material. Protein seem to be excluded from these considerations because pronase did not diminish the activity of either NE or TE; contrariwise, as documented in our results, such treatment frequently enhanced the activity of the extracts. This may still imply association of a residual protein with our active principle. Treatment with RNAase always led to partial destruction of activity. The association of DNA and RNA sequences in a fully active extract is therefore a viable possibility, and we intend to investigate this directly. The material can be prepared in large quantities by a modification of the Perry extraction technique

[20], which yields much more DNA than our present method. More difficult but probably less destructive to the presumptive DNA–RNA complex would be a preparative slab acrylamide gel technique designed by our colleague, Sidney Altman. With such technique, we should be able to purify the slower moving peak by electrophoretic elution. Hopefully this will yield quantities of the active fraction sufficiently large for biochemical and biological analysis.

EXPERIMENTAL

Seeds of *Phaseolus vulgaris* L. cv. Astro obtained from Asgrow (Orange, CT, U.S.A.) were planted in vermiculite and subirrigated twice daily with a solution of Hyponex (Hydroponics Chemicals Co., Copley, OH, U.S.A.; 1·2 g/l.). Plants were grown under 16 hr/day (8 a.m.–12 p.m.) of *ca* 43000 lx fluorescent–incandescent light (9:1 energy ratio) at 24° and *ca* 75% r.h. in environmental growth chambers. Crown galls were induced by administering 7-mm-long wounds to 4–6-day-old hypocotyls with a sterile scalpel; inocula from 24–48-hr-old slant cultures of *Agrobacterium tumefaciens* var. T 37 (supplied by Dr Armin C. Braun, Rockefeller University) were then applied to the wound. Tumors could be harvested and freed of normal tissues 28–60 days later. For donor nucleic acid preparations either tumors or 8-day-old normal hypocotyl tissues were washed, weighed, frozen over solid CO_2 and extracted with phenol by the method of Loening and Ingle [21], with or without 10 mM mercaptoethanol. The aqueous phase, containing the nucleic acids, was extensively dialyzed against Tris–HCl buffer, pH 7·4 in the cold and made 2% in KOAc; the nucleic acids were then precipitated by the addition of 2·5 vol. cold EtOH. After extensive washing with EtOH, the precipitate was redissolved in 10 mM Tris buffer, pH 7·4. The absorption spectrum of such preparations was typical for nucleic acids and showed an $A_{260:280}$ ratio of more than 2·0; the acrylamide gel electrophoresis patterns [22] when scanned at 260 mm showed a small DNA peak near the origin, the usual four plant *r*-RNA peaks and a small peak of low-molecular weight, rapidly moving material.

Receptor tissues of 3-day-old normal hypocotyl and crown galls were surface-sterilized, excised, washed several times with sterile, deionized H_2O and immersed in 10 mM Tris–HCl buffer, pH 7·4 either containing or lacking donor extracts. Normal and tumorous extracts (NE and TE, respectively) were added at concentrations of 33–1333 μg/ml (measured as 1·0 absorbance unit at 260 nm = 40 μg RNA/ml). Most experiments were performed under aseptic conditions; in some cases penicillin (100 units) and streptomycin (100 μg/ml) were added to suppress bacterial growth. The donor solutions containing immersed receptor tissue segments were placed in sterile, foil-covered beakers in a desiccator and vacuum infiltrated at room temp for 25 min. After release of the vacuum, the tissues were gathered and postincubated for 12 hr by shaking in sterile Heller's medium [23] containing or lacking radioactive amino acids. The tissues were then washed, blotted, weighed, quickly frozen and stored at −16° until protein extraction.

Soluble proteins were extracted by the van Loon–van Kammen technique [24]. Peroxidase activity determination, isoperoxidase separations on starch gel and their subsequent visualization and quantitation were done by previously published methods [25]. In experiments involving the effect of NE and TE on incorporation of labelled amino acids (0·5–1·0 μCi/ml ^{14}C-leucine or ^{14}C-labelled mixed amino acids, New England Nuclear Co.), both TCA-soluble and -insoluble counts were determined, and the results expressed as incorporation efficiency (IE), which is the ratio TCA insoluble/soluble counts. Protein content was determined by the Lowry method [26] using bovine serum albumin (Sigma Chem. Comp., Fraction V) as a standard.

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