Plant Physiol. (1973) 52, 368–372

Chromatin-directed Ribonucleic Acid Synthesis

A COMPARISON OF CHROMATINS ISOLATED FROM HEALTHY, AVIRULENT *AGROBACTERIUM TUMEFACIENS* INOCULATED, AND CROWN-GALL TUMOR TISSUES OF *VICIA FABA*

Received for publication April 2, 1973

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ABSTRACT

Chromatin was extracted from healthy, avirulent *Agrobacterium tumefaciens* inoculated, and crown-gall tumor *Vicia faba* internodes of the same age. Chromatin from crown-gall tissue produced 5 times more RNA per 100 micrograms of DNA than chromatin from the healthy tissue. When template availability was compared using chromatin with saturating amounts of *Escherichia coli* RNA polymerase, chromatin from crown-gall tissue had 36% more available template than the controls. In addition, when γ-^{32}P-ATP was incorporated into the RNA synthesizing reaction mixture, with saturating amounts of *E. coli* RNA polymerase, there were twice as many RNA chain starts in tumor as in control tissue.

Crown-gall disease, incited by the bacterium *Agrobacterium tumefaciens* (Erwin F. Smith and Touns) Conn., is characterized by the production of autonomously growing plant tumors. Although there has been a large amount of research done with this disease (2–4), the exact mechanism of tumor induction remains unknown.

A. tumefaciens induces new biosynthetic pathways in host tissue (28). To account for these new pathways which result in autonomous growth of tumor cells, three hypotheses have been advanced. The first proposes that the virulent strain of *A. tumefaciens* carries a phage which is responsible for tumor development (18). After the bacterium enters the plant, it releases the phage which then transforms normal plant cells into tumors. This hypothesis receives support from investigations in which a bacteriophage active against *A. tumefaciens* was isolated from sterile sunflower gall tissue (18). Further evidence suggests that the DNA from such a phage is capable of inducing tumors in the plant species from which it was isolated (13). However, electron microscopy studies of plant tumor cells have detected no bacteriophage or viral-like structures (11, 14), and buoyant densities and melting temperatures of the DNA from both virulent and avirulent strains of *A. tumefaciens* are virtually identical (12).

According to the second hypothesis, the DNA of the bacterium is incorporated into the host genomes (21, 26). Hybridization studies between bacterial DNA, crown-gall tumor cell DNA, and normal cell DNA reveal greater homology between bacterial and tumor cell DNA than between bacterial and normal cell DNA (23). Shearing tumor cell DNA releases a very small fragment which has the same buoyant density as *A. tumefaciens* DNA (25). It was interpreted that this fragment represents bacterial DNA incorporated into the host genome.

Because a new RNase (19) and new hydrolases (8) not characteristic of either normal plant cells or *A. tumefaciens* were found in crown-gall cells, a third hypothesis suggests that derepression of host genes may be responsible for tumorigenesis (2, 28). Research in this area is rather meager, probably because the mechanism of genetic regulation in eucaryotic cells remains largely unknown.

Since all these hypotheses involve the nucleic acid metabolism of the host, examination of some of the properties of tumor cell chromatin should provide useful information for solving the problem of tumorigenesis. The purpose of this research was (*a*) to characterize chromatin from healthy, avirulent-inoculated and tumorous tissues in relation to DNA-dependent RNA polymerase activity, (*b*) to compare the amount of DNA template available for transcription in the extracted chromatins, and (*c*) to measure the number of RNA chain starts and mean chain lengths produced by the chromatins *in vitro*.

MATERIALS AND METHODS

Vicia faba L. seeds (J. Harris Co., Inc., Rochester, N.Y.) were planted at a uniform depth in a soil-sand-peat mixture (3:2:1), and kept in the greenhouse at 25 C ± 3. When seedlings reached the height of 20 cm (about 2 weeks), they were divided into three groups. One group was inoculated by needle puncture at 12 separate sites in the first two internodes with a 48-hr culture of *A. tumefaciens* strain 806 (obtained from Dr. T. T. Stonier) grown at ambient temperature in nutrient broth plus 0.5% glucose; the second group was inoculated in the same manner with a 48-hr culture of *A. tumefaciens* 806 avirulent grown as above; the third group was untreated, but the first two internodes were marked for later reference.

Chromatin Extraction. Mature tumors were formed 4 weeks after inoculation, at which time the internodes from all three groups were excised. Chromatin was extracted by the method of Huang and Bonner (9) as modified by O'Brien *et al.* (17). The tissue was weighed in ice-cold beakers, minced and homogenized in buffer A (50 mM tris-HCl, pH 8.0; 1 mM $MgCl_2$; 0.25 M sucrose; 20 mM β-mercaptoethanol) for 1 min at high speed in a Waring Blendor. A buffer-tissue ratio of 2:1 (v/w) was used for all chromatin extractions. After filtering through four layers of cheesecloth and one layer of Miracloth, the suspension was centrifuged at 10,000*g* for 30 min. The gelatinous pellet was scraped from the underlying starch, suspended

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in buffer B (10 mM tris-HCl, pH 8.0; 0.25 M sucrose; 10 mM β-mercaptoethanol) and pelleted twice (10,000*g* for 30 min). Following the last centrifugation, the pellet was suspended in 5 ml of buffer B and solubilized by use of a glass homogenizer. The solution was layered over 20 ml of 1.8 M sucrose in 10 mM tris-HCl buffer, pH 8.0 and 10 mM β-mercaptoethanol. The top third of the tubes were stirred forming a rough gradient and centrifuged at 40,000*g* for 3 hr. After resuspending the pellet in 5 ml of 10 mM tris-HCl, pH 8.0, containing 10 mM β-mercaptoethanol, a 2-ml aliquot was dialyzed against the same buffer with three changes to remove the sucrose. A diphenylamine test (5) was performed with a perchloric acid digest of the dialyzate to determine DNA content (1 part 0.5 N $HClO_4$:1 part sample, heated at 70 C for 30 min). Calf thymus DNA (Worthington Co., Freehold, N.J.) was used as a standard for the diphenylamine test. Except as indicated, all of the above steps were carried out at 0 to 4 C.

DNA-dependent RNA Polymerase Assay. To measure chromatin-bound RNA polymerase activity, 0.1-ml aliquots of chromatin, containing about 20 μg of DNA from each sample, were added to 0.25 ml of a cold reaction mixture containing (in μmoles) tris-HCl (pH 8.0 at 25 C), 10; $MgCl_2$, 1.0; $MnCl_2$, 0.2; β-mercaptoethanol, 3.0; GTP, CTP, and UTP, 0.1; and 0.1 μc of ^{14}C ATP (New England Nuclear, 32.5 mc/mmole). Reactions were carried out in duplicate at 25 C, rather than at a higher temperature, to minimize RNase activity which is present in the chromatin preparation. Each reaction was stopped by the addition of 2 ml of cold 10% trichloroacetic acid containing 0.9% sodium pyrophosphate. A zero time value was obtained by adding trichloroacetic acid to a cold reaction mixture. Trichloroacetic acid-insoluble material was recovered on membrane filters (Bac-T-Flex, B-6; Schleicher and Schuell, Keene, N.H.), and washed with 30 ml of ice-cold 10% trichloroacetic acid containing 0.9% sodium pyrophosphate. After drying, the filters were placed in Liquifluor scintillation fluid (New England Nuclear), and radioactivity was assayed with a Unilux I Nuclear Chicago liquid scintillation counter which had a 60% efficiency for this particular assay.

The amount of template available for transcription was measured in a similar manner, except that 0 to 15 units of *Escherichia coli* RNA polymerase (Sigma Chemical Co., St. Louis, Mo.) were added to the reaction mixture, and the reaction was terminated after 20 min at 25 C. The amount of chromatin DNA added to the reaction mixture was reduced to the region of 0.5 μg, so that the *E. coli* polymerase could saturate the available template within a reasonable number of units. Endogenous polymerase activity (no *E. coli* enzyme added) of the chromatins at this DNA concentration was measured, and the radioactivity was subtracted from the experimental values.

Inhibition of DNA-dependent RNA Polymerase. In order to ensure that RNA was the product of the polymerase reaction, Actinomycin D (Sigma Chemical Co.) and RNase A (Sigma Chemical Co.) were added to the reaction mixtures. In the chromatin-bound polymerase assay, 0.2 μg/ml Actinomycin D or 10 μg of RNase A were added to the reaction mixtures. The effect of deleting $MnCl_2$ and $MgCl_2$ was also tested. Reaction mixtures were stopped after 20 min, and the incorporation of ^{14}C ATP into the trichloroacetic acid-insoluble fraction was measured as described above.

Since chromatin-directed RNA synthesis may be affected by DNase and RNase associated with the chromatins, the activity of these enzymes was measured. One ml of chromatin (100 μg) was added to 2 ml of 0.2% yeast RNA (Sigma Chemical Co.) or calf thymus DNA (Sigma Chemical Co.). The reaction was carried out at 37 C for 4 hr and terminated by adding 3 ml of 10% trichloroacetic acid. After precipitating for 5 hr at 4 C, the mixtures were centrifuged at 20,000*g* for 30 min and the A_{260} of the supernatant *versus* a zero time control was read.

Assay for RNA Chain Starts. The number of RNA chain starts was measured using the assay devised by Maitra and Hurwitz (15). Essentially, the assay is the same as the one used to measure chromatin-bound RNA polymerase activity, except 0.4 μM of γ-^{32}P-ATP (specific radioactivity 5.5 c/mmole; New England Nuclear Corp.) was added in place of ^{14}C ATP, and a saturating amount of *E. coli* RNA polymerase was also added. The control for this experiment was to incubate the chromatin in the same volume of reaction mixture but without any components except the γ-^{32}P-ATP. The resulting radioactivity was then subtracted from the experimental values. This control was necessary to show that the radioactivity was not due to phosphorylation of histones and other nuclear proteins by protein kinases. A simultaneous assay with the same components, except replacing γ-^{32}P-ATP with ^{14}C ATP, was run to measure total RNA production.

All experiments were performed in duplicate on three separate chromatin extractions from each type of tissue. The data are expressed as the mean ± the standard error of the mean.

RESULTS

In order to ensure that plant chromatin was not contaminated with chromatin or DNA from *A. tumefaciens*, preliminary experiments were performed which indicated that the grinding conditions used to extract chromatin disrupted very few bacterial cells. Also penicillin G and streptomycin sulfate were added to all buffers and reaction mixtures at a concentration of 0.1 mM. Plating of plant chromatin in Bacto-nutrient agar during various phases of its purification showed no viable organisms.

Chromatin directed RNA synthesis is sensitive to Actinomycin D, an inhibitor of DNA-dependent RNA polymerase (Table I). The reaction mixture containing Actinomycin D resulted in at least 93% reduction in the amount of ^{14}C ATP incorporated into the trichloroacetic acid-insoluble fraction. RNase A was used to show that the labeled nucleoside triphosphate was actually incorporated into RNA (at least 82% reduction of ^{14}C incorporation). The reaction is also dependent

Table I. *Effect of Various Assay Conditions on the Incorporation of ^{14}C ATP into RNA by Chromatins from Healthy, Avirulent-inoculated, and Tumorous Vicia faba Tissues*

Assay Conditions	ATP Incorporated		
	Healthy	Avirulent-inoculated	Tumorous
	pmoles ^{14}C/20 min·100 mg DNA		
Complete	11.8 ± 1.2 (100)[1]	17.0 ± 1.4 (100)	60.0 ± 1.6 (100)
$-Mg^{2+}$	5.5 ± 0.8 (46)	9.1 ± 0.8 (54)	32.0 ± 0.6 (53)
$-Mn^{2+}$	5.0 ± 0.6 (42)	7.6 ± 0.9 (45)	24.4 ± 0.2 (41)
$-Mg^{2+}$ $-Mn^{2+}$	1.2 ± 0.4 (10)	1.5 ± 0.3 (8)	5.4 ± 0.4 (9)
+0.2 M KCl	2.1 ± 0.3 (18)	2.5 ± 0.6 (15)	9.9 ± 0.6 (16)
+150 μg/ml Rifamycin	11.7 ± 1.3 (99)	16.6 ± 1.2 (98)	46.8 ± 1.3 (78)
+0.2 μg/ml Actinomycin D	0.6 ± 0.4 (5)	1.2 ± 0.5 (7)	3.6 ± 0.5 (6)
+10 μg/ml RNase	2.1 ± 0.3 (18)	2.8 ± 0.2 (16)	10.8 ± 0.9 (18)
+1.0 mM $NaPO_4$	11.6 ± 1.2 (98)	16.4 ± 1.3 (96)	57.9 ± 3.0 (96)
+1.0 mM Pyrophosphate	2.2 ± 0.2 (19)	3.0 ± 0.4 (18)	9.6 ± 0.4 (16)

[1] Numbers in parentheses indicate percentage of control.

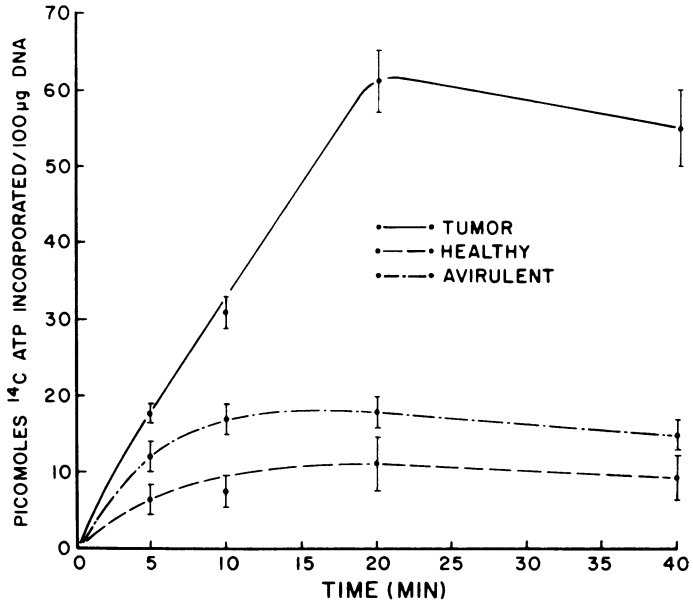

FIG. 1. Comparison of rates of RNA synthesis *in vitro* using 20 µg of chromatin isolated from healthy, avirulent-inoculated, and crown-gall tumor broad bean internodes. See text for details of reaction mixtures.

upon the presence of both Mg^{2+} and Mn^{2+} ions, inhibited by 0.2 M KCl and 0.1 mM pyrophosphate, but not by 0.1 mM inorganic phosphate. These data indicate that the product of the chromatin reaction mixture is predominantly RNA.

Rifamycin, a specific inhibitor of *A. tumefaciens* RNA polymerase (unpublished results), had no effect on RNA synthesis of the controls, but did inhibit slightly and consistently (22%) RNA synthesis by tumor chromatin. There are a number of possibilities which may account for this observation. It may be possible that trace amounts of bacteria, while not able to multiply because of the penicillin and streptomycin, were able to synthesize RNA and thus would be sensitive to a rifamycin treatment. Another possibility is contamination of the chromatin with extranuclear RNA polymerases (mitochondrial or chloroplast) which may be sensitive to rifamycin. This is less likely, however, since the two controls should also be contaminated. A final explanation might be that a fraction of the bacterial DNA which is responsible for coding the production of bacterial RNA polymerase is present in the tumor chromatin. If this were the case, then some of the bacterial RNA polymerase would probably remain attached to the chromatin during its purification.

Alterations in the composition of the grinding medium, ratio of grinding medium to weight of plant tissue, and method of grinding resulted in variable losses of chromatin-bound polymerase activity. The recovery of DNA chromatin from tumorous plants varied from 26 to 30 µg DNA/g fresh weight depending on the size of the galls. Chromatin from avirulent-inoculated and healthy tissues ranged between 17 and 18 µg DNA/g fresh weight.

Production of RNA by chromatin-bound RNA polymerase of healthy, avirulent-inoculated and crown-gall tissue is illustrated in Figure 1. Although the absolute maximum product was synthesized after 20 min by chromatin from all three tissue types, statistically there is no difference between the amount of RNA produced at 10 and 20 min for both of the control chromatins. The enzyme reaction rate of all three tissues levels off after 20 min and the amount of RNA synthesized at that time by tumor chromatin is three times that of the inoculated control and five times greater than the healthy control. Also the RNA produced by the inoculated control is significantly greater than the healthy control.

Since RNA synthesis could be influenced by RNase and DNase enzymes associated with the chromatins, these variables were measured (Table II). There is no significant difference between the RNase activity of healthy chromatin and tumor chromatin or avirulent-inoculated chromatin and tumor chromatin.

RNA production may be affected by DNase because RNA polymerase molecules seem to attach readily to "nicked" portions of DNA (27). The DNase activity of all three chromatin preparations also overlap. In addition, if the RNase and DNase reactions were carried out at 25 C instead of 37 C, no activity could be detected in any of the preparations, even after 24-hr incubation.

For optimum synthesis of RNA, all three chromatins require the presence of both Mg^{2+} and Mn^{2+} ions. The data in Figures 2 and 3 show the optimum ion concentration of both to be 20 to 30 mM. Note that even though the single ion concentration is greater than the combined (Mg^{2+} and Mn^{2+}) ion concentration used in the kinetic experiment (Fig. 1), the amount of RNA synthesized is less, verifying the need for two separate metal ions for maximum enzyme activity.

Since the increase in RNA synthesis by tumor chromatin

Table II. *RNase and DNase Activity Associated with Chromatin from Healthy, Avirulent Agrobacterium tumefaciens Inoculated, and Tumor Tissue of Vicia faba*

	Specific Activity of Enzyme		
	Healthy	Avirulent	Tumor
	units[1]/mg of DNA or RNA		
RNase	0.02 ± 0.02	0.15 ± 0.1	0.05 ± 0.05
DNase	0.8 ± 0.5	2.3 ± 1.6	2.7 ± 1.7

[1] One enzyme unit represents an increase of 1.0 A_{260} for an incubation period of 4 hr.

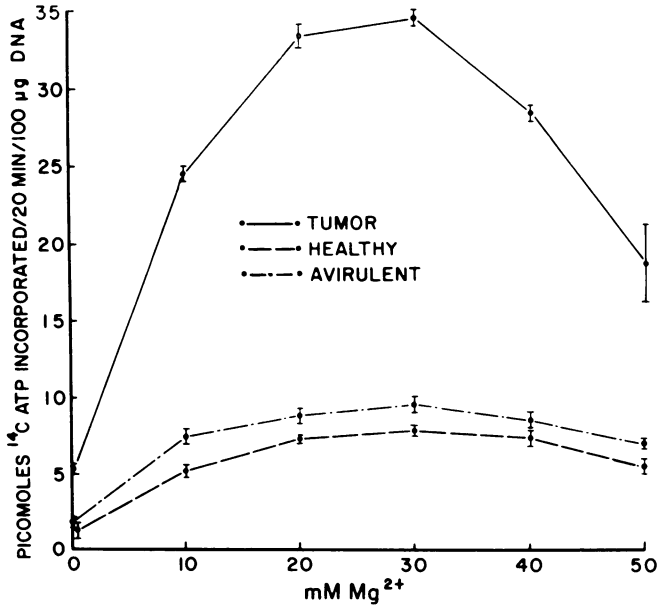

FIG. 2. Effect of Mg^{2+} on chromatin-bound RNA polymerase activities. Assay conditions were as described in text except for the metal-ion concentrations.

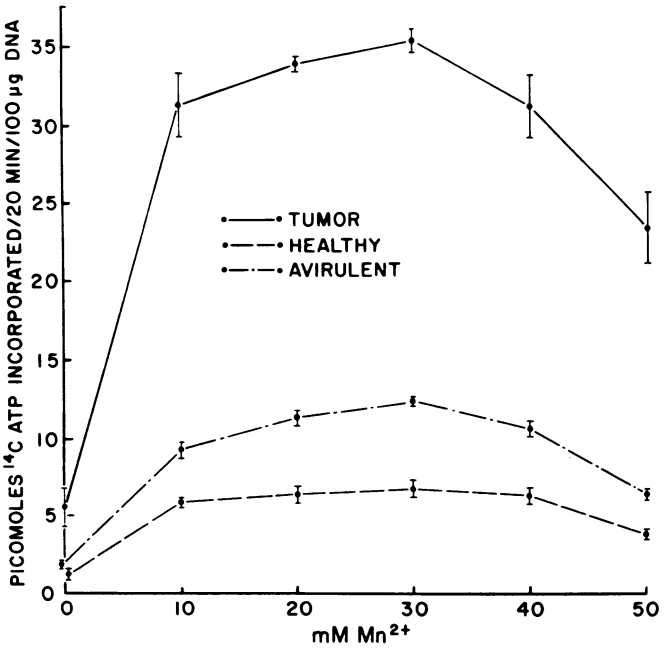

FIG. 3. Effect of Mn^{2+} on chromatin-bound RNA polymerase activities. Assay conditions were as described in text except for the metal-ion concentrations.

could be due either to a greater amount of chromatin-bound RNA polymerase or to more genetic sites available for transcription, a reaction was run using increasing amounts of *E. coli* RNA polymerase with 0.5 µg of chromatin as template to distinguish between the two possibilities.

RNA production by fortified chromatin (chromatin with *E. coli* polymerase added) of healthy, avirulent-inoculated and tumor tissue is presented in Figure 4. Saturation was achieved with the same number of units of polymerase (5 units) in all three chromatins. At saturation the two controls yield about the same amount of RNA, however, fortified tumor chromatin produced about 36% more RNA. These data suggest that there are approximately 36% more sites available for transcription in tumor DNA than DNA from the controls. At higher concentrations of *E. coli* RNA polymerase there is a slight decrease in RNA synthesis in all three samples. This is probably due to contaminating traces of RNase in the commercial preparation of *E. coli* RNA polymerase used.

By using γ-^{32}P-ATP, it is possible to estimate the number of RNA chains starting with ATP since the γ phosphate is retained only in the initial nucleotide (15). Combining these data with a measurement of total RNA synthesis by using ^{14}C ATP, it is possible to arrive at a mean RNA chain length. Although the mean tumor RNA chain length is significantly smaller than the two control RNA lengths, the significant difference (10.6%) is less than the significant difference between the control and tumor γ-^{32}P-ATP incorporation (30.0%). Thus it is safe to assume that the data (Table III) indicate that the increase in RNA synthesis by fortified tumor chromatin (chromatin saturated with *E. coli* polymerase, Fig. 3) is due to an increased number of RNA chains. Since *E. coli* RNA polymerase starts RNA chains with either ATP or GTP (15), it is not possible to deduce the total number of RNA chain starts from these data. Also, it is not known whether these additional RNA chains represent new genetic information.

Although the total RNA produced by the three fortified chromatin preparations (Fig. 4) is less than the total RNA produced in the chain initiation experiments (Table III), the relative differences among the chromatins remain the same. This variation in total RNA production may be attributed to the time of year that the control and tumor plants for the different experiments were grown and harvested.

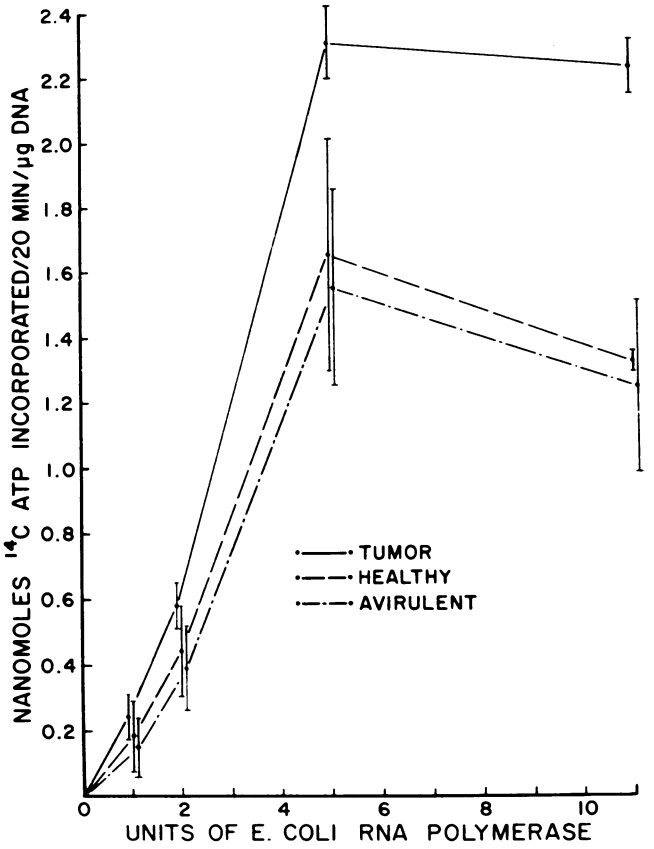

FIG. 4. Comparison of rates of RNA synthesis *in vitro* following addition of *E. coli* RNA polymerase to a reaction mixture containing 0.5 µg of chromatin isolated from healthy, avirulent-inoculated, and crown-gall tumor broad bean internodes. See text for details of reaction mixtures.

Table III. *Incorporation of γ-^{32}P-ATP to Determine Mean RNA Chain Lengths in vitro using Chromatins from Healthy, Avirulent-inoculated, and Tumorous Vicia faba Tissues as Templates*

Template	RNA Synthesized[1]	γ-^{32}P-ATP Incorporated	Mean RNA Chain Length
	nmoles	*pmoles*	
Healthy	25.9 ± 1.3	2.3 ± 0.1	11,261 ± 799
Avirulent inoculated	28.6 ± 0.8	2.4 ± 0.4	11,917 ± 1417
Tumor	41.5 ± 2.8	4.8 ± 0.8	8,646 ± 735

[1] Total RNA was calculated by multiplying ^{14}C ATP nmoles by 3.85 (15).

DISCUSSION

The results indicate that the capacity of tumor chromatin to synthesize RNA is greatly increased. This finding verifies earlier work which showed higher ^{32}P incorporation into RNA by tobacco crown-gall callus (22). A small part of this increase can be explained by a greater number of available transcription sites in tumor DNA as shown by increased number of RNA chains.

An increase in genetic sites may be accomplished in a number of ways. Genes in the host may be activated (derepressed) by a substance elicited or induced by the bacterium. Histones, which are thought to play a role in genetic regulation (1, 6), when applied to a plant within 4 days after inoculation, cause inhibition of tumor development (7). Another possibility is the incorporation of a viral genome or *A. tumefaciens* DNA into the genome of the host cell, thus increasing the genetic potential of the host, provided that the chromatin-bound RNA polymerase could recognize and transcribe the foreign DNA.

The remaining increase in RNA synthesis ability of tumor chromatin cannot be explained by differences in RNase and DNase activities, and therefore, it seems reasonable to assume that tumor chromatin has a larger amount of endogenous RNA polymerase or that the tumor RNA polymerase is modified, enabling it to transcribe more effectively. The small increase in RNA synthesis by the chromatin from avirulent-inoculated tissue over chromatin from untreated tissue (Fig. 1) may be due to an increase in RNA polymerase synthesis induced by wounding. In fact, in preliminary experiments with chromatin from sterile wounded tissue, small increases in RNA synthesis were also observed.

Research similar to that reported here has been carried out using tobacco callus tissue and tobacco crown-gall callus tissue (24). No difference was found in the chromatins from the two types of callus tissue with regards to chromatin-bound RNA polymerase activity or amount of template available for transcription. However, many growth compounds must be added to a culture medium in order to obtain a callus tissue from healthy plants. Some of these compounds have been shown to change some properties of plant chromatin (10, 16, 17, 20). Therefore, differences between healthy and tumor chromatins may be masked when using callus tissue.

Although it is obvious that there are marked differences between crown-gall and healthy tissue chromatins, it is not possible to tell from the data whether the changes are due directly to transformation by the tumor inducing principle (3) or to secondarily accumulated growth compounds. Studies on the changes in plant chromatin over a time period following inoculation should clarify this latter point.

Acknowledgments—The authors wish to thank Dr. T. T. Stonier, Manhattan College, N.Y., for providing the *Agrobacterium tumefaciens* cultures used in this study, and also Dr. Peter Parsons, Assistant Professor of Biochemistry, University of Massachusetts, Amherst, and Dr. Karl Hittlemann, Assistant Professor of Biochemistry, University of Massachusetts Medical School, Worcester, for suggestions in preparation and for critical review of the manuscript.

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