Transcription of Walker 256 Carcinosarcoma Chromatin

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SUMMARY

The in vitro transcriptions from chromatins of Walker 256 carcinosarcoma, rat mammary gland, and rat liver were compared with respect to template activities and reciprocal double saturation hybridization of the transcribed RNA's. Walker tumor chromatin was found to have the least template activity in RNA synthesis in vitro, and the synthesized RNA, when annealed with DNA, also showed the least saturation hybridization plateau. Hybridization studies further showed the presence of new RNA species in transcripts from Walker tumor chromatin which were not detected in normal in vitro chromatin transcription. These results indicate that the transformation from normal to neoplastic state both repression and derepression of normal transcribable DNA sequences occur.

INTRODUCTION

Hepatoma chromatin contains repressed cistrons that are present in chromatins of related normal tissues (1, 40). It has also been reported (8) that Novikoff hepatoma cells contain 167% as much heterochromatin as do normal liver cells. This evidence indicates a state of suppressed genetic activity in neoplastic cells. Recent reports, however, have shown rapidly labeled new RNA species synthesized in mammary carcinoma and in chronic lymphocytic leukemia cells which are not detected in normal cells (10, 39). These findings would support the view that the transformation from the normal state to cancer involves a derepression of potentially oncogenic genes. This seeming contradiction could be explained if gene expression in cancer is assumed to involve both repression and derepression of normal genome sequences. One approach to resolving this problem is to measure simultaneously the restricted and additional transcribable genetic activities expressed in normal and tumor chromatins. In this work, a comparison was made of the in vitro transcription of chromatins from Walker 256 carcinosarcoma, rat mammary gland, and rat liver. Data will be presented to show that the tumor chromatin has both a more restricted transcription and also additional transcribable sequences than normal chromatins. These results are consistent with the belief that both repression and derepression of normal genetic activities play a part in cancer.

MATERIALS AND METHODS

Animals. All rats used in this investigation were of the Sprague-Dawley strain and were purchased from Holtzman Company, Madison, Wis. Livers were obtained from female rats, and abdominal mammary glands were from female rats 15 to 20 days pregnant. The Walker 256 carcinosarcoma was transplanted to the flanks of female rats and harvested 10 days postinoculation. This transplantable tumor, derived from rat mammary gland, is histocompatible with the original host. Some metastasis is associated with the lymph nodes since the original passage in 1928 (35). The similarities of the tumor and liver cells have been reported by Molnar and Bekesi (25).

Preparation and Fractionation of Chromatin. All procedures were carried out in a cold room at 4°C. Cell nuclei were isolated from Walker tumor, mammary gland, and liver by a modification of the method of Chauveau et al. (7) with precautions taken as described by Busch and Steele (5). The isolated nuclei were successively extracted with 0.14 M NaCl in 0.05 M Tris-HCl, pH 8.0, with the aid of a Dounce homogenizer. Chromatin was prepared from the extracted nuclei according to the method of Seligy and Miyagi (31). The pelleted chromatin was washed free of sucrose with 0.05 M Tris-HCl, pH 8.0, and suspended in distilled water overnight. The swollen chromatin was adjusted to a DNA concentration of 200 μg/ml for use in the experiments to be described. DNA content of the chromatin was determined by the method of Burton (3).

Isolated chromatin of known DNA concentration was extracted 3 times with 0.14 M NaCl in 0.05 M Tris-HCl, pH 8.0, to rid the chromatin of soluble proteins. The chromatin was then extracted with 2.0 M NaCl in 0.02 M Tris-HCl, pH 8.0. The 2.0 M NaCl mixture was centrifuged at 30,000 rpm in a Spinco No. 30 rotor for 1 hr. Supernatant solution was set aside for processing to be described later; the pellet (salt insoluble) was solubilized to yield the residual nonhistone proteins as described previously (41). The supernatant extract (salt soluble) was dialyzed against 13 volumes of water to precipitate the DNA-histone. The DNA-histone was pelleted at 30,000 rpm for 1 hr in a Spinco No. 30 rotor, while the supernatant solution contained the salt-soluble nonhistone proteins (43). Histones were obtained from the DNA-histone pellet by 3 successive extractions with 0.20 M HCl. The combined extract which contained the histones was dialyzed thoroughly against water. Protein concentration was determined by the procedure of Lowry et al. (21).

Polyacrylamide gel electrophoresis of the nonhistone proteins was performed as described previously (18) except that in the case of the salt-soluble nonhistone proteins, the running buffers were supplemented with 4 M urea and 0.1% SDS.

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The abbreviations used are: SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; 1 X SSC, 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0.
Preparation of *Micrococcus lysodeikticus* RNA Polymerase. *M. lysodeikticus* RNA polymerase was prepared according to the procedure of Nakamoto et al. (26). Specific activities of the diethylenoethyl Fraction VI RNA polymerase preparations ranged from 500 to 700 units/mg of protein, as determined by the assay procedure of Nakamoto et al. (26).

**Template Activity of Chromatin.** For determination of the template activities of the chromatin, the standard reaction mixture of Nakamoto et al. (26) was used except that UTP-3H replaced 32P-labeled ribonucleoside triphosphate and calf thymus DNA was replaced by chromatin as the template. The reaction mixture, in a volume of 0.5 ml, was incubated at 30° for 10 min. At the end of the incubation, the reaction was terminated by chilling in ice water, and 0.1 ml of cold 50% TCA and 2 ml of cold 5% TCA were added in succession. The mixture was mixed after each addition of TCA. The acid-insoluble precipitate was collected on a Millipore filter (0.45 μ) and washed 5 times with 5-ml portions of cold 5% TCA. The filter was dissolved in 10 ml of scintillation fluid consisting of 10% naphthalene, 0.7% PPO, and 0.03% POPOP in dioxane, and radioactivity was counted in a Packard scintillation spectrometer.

**Isolation of DNA.** DNA was isolated from the nuclei of Walker tumor, rat liver, and mammary gland by a modification (24) of the method of Marmur (22). Nuclei were suspended in 0.25 M sucrose containing 0.1 M EDTA, pH 8.0 and 0.14 M NaCl. SDS was added to the suspension to 0.5%, and the suspension was extracted twice with a chloroform-isooamyl alcohol (24:1) mixture. After centrifugation, the nucleic acids were precipitated from the aqueous phase with ethanol. The crude DNA was collected on a stirring rod and dissolved in 0.01 X SSC. Once in solution, the salt concentration of the solution was raised to 2 X SSC by addition of 10 X SSC. This mixture was treated with RNase (50 μg/ml) at room temperature for 1 hr, followed by digestion with autodigested Pronase (1 mg/ml). The Pronase was allowed to react for 4 to 6 hr. The solution was made to 0.05 M Tris-HCl, pH 8.0, and 0.5% SDS. This DNA solution was then extracted alternately with chloroform-isooamyl alcohol and water-saturated phenol until there was no precipitate in the interphase. The DNA was precipitated from the aqueous phase by addition of 0.54 volume of isopropyl alcohol, collected on a glass rod, and washed in ethanol. The DNA was then dissolved in 0.01 X SSC for use.

**Isolation of RNA Synthesized in Vitro.** The *in vitro* synthesis of RNA with various chromatin as template was carried out according to the procedure of Tan and Miyagi (37). In order to synthesize a sufficient quantity of RNA, the standard reaction mixture for RNA polymerase reaction was increased 40-fold. The reaction mixture, in a final volume of 10 ml, contained: Tris-HCl, pH 7.5, 500 μmoles; RNA polymerase, 1000 units; 3H-labeled ribonucleoside triphosphates of adenine, guanine, cytosine, and uracil, 10 μmoles (0.125 mCi) each; MnCl2, 25 μmoles; spermidine phosphate, 20 μmoles; and chromatin equivalent to 1 to 1.5 mg of DNA. The reaction mixture was incubated for 30 min, and an additional 500 units of RNA polymerase were added to the reaction mixture. The reaction was allowed to proceed for 30 min. At the end of incubation, SDS and NaCl were added to the mixture to a final concentration of 0.5% and 0.14 M, respectively. The nucleic acids were extracted with water-saturated destilled phenol at room temperature. After 2 extractions with phenol, the nucleic acids were precipitated from the aqueous phase by addition of 2 volumes of cold 90% ethanol, allowed to stand at −20° for 2 hr, and collected by centrifugation. The nucleic acid pellet was dissolved in 0.01 M Tris-HCl, pH 7.5, containing 0.01 M MgCl2 and digested with DNase (50 μg/ml) at room temperature for 1 hr. NaCl and SDS were then added to the mixture to 0.14 M and 0.5%, respectively, and the mixture was extracted with phenol until there was no precipitate in the interphase. The RNA was again precipitated from the aqueous phase with ethanol, dissolved in 0.01 X SSC, and dialyzed against 3 liters of the same solvent with 6 changes of dialysate over a 24-hr period. The synthesized RNA had a ratio of absorbance at 260 μm to that at 280 μm of 1.9 and to that at 230 μm of 2.8. The specific radioactivity of the *in vitro*-synthesized RNA was 2 to 3 X 104 cpm per μg of RNA.

**Hybridization of RNA Synthesized in Vitro with DNA.** DNA was denatured by alkali, and 1 μg of the denatured DNA was immobilized on 25-mm nitrocellulose membrane filters (Schleicher and Scheull, Type B-6) according to the method of Gillespie and Spiegelman (13). Annealing was carried out essentially by the procedure of Tan and Miyagi (37). A reaction mixture of 1 ml containing the respective amounts of RNA in 30% formamide and 2 X SSC was incubated at 37° for 24 hr in screw cap vials. Each vial contained 2 DNA filters and 2 blank filters. At the end of the 24-hr incubation period, 1 DNA filter with a saturating amount of RNA and 1 blank filter were transferred to increasing amounts of competing RNA. The double saturation was allowed to proceed for an additional 24 hr of incubation. At the end of the initial and 2nd 24-hr incubation, the annealed and blank filters were prepared for counting according to the method of Gillespie and Spiegelman (13). The background “noise” was approximately 0.005% of the input RNA counts.

**RESULTS**

Some Properties of the Chromatins of Walker Tumor, Rat Liver, and Mammary Gland. Quantitative estimation of salt-soluble nonhistone proteins of Walker tumor, rat liver, and rat mammary chromatin yielded weight ratios of 1.05, 0.65, and 0.55; those of salt-insoluble proteins yielded ratios of 1.24, 0.33, and 0.27; and those of histones yielded ratios of 1.54, 0.90, and 0.91, per unit weight of DNA, respectively. This analysis is in agreement with results obtained by other investigators (9, 12, 15, 34, 47, 48) who reported a higher percentage of nonhistone proteins in tumor than in normal tissues. Further qualitative differences among the nonhistone proteins of Walker tumor, rat liver, and mammary gland are shown by their electrophoretic patterns in Figs. 1 and 2.

When the isolated chromatins were incubated with 3H-labeled RNA for 1 hr (31), there was no detectable TCA-soluble radioactivity. Intact chromatins therefore do not manifest measurable RNase activity.

**Template Activities of Chromatins of Walker Tumor, Rat Liver, and Rat Mammary Gland.** When assayed in *M. lysodeikticus* RNA polymerase system, the Walker tumor...
Fig. 1. Polyacrylamide gel electrophoresis of the salt-soluble nonhistone proteins isolated from rat liver (1), rat mammary gland (2), and Walker tumor (3). Samples of approximately 200 µg of protein were used for each of the runs. Electrophoresis was from top (cathode) to bottom (anode) as described previously (18) except that the running buffers were supplemented with 0.1% SDS and 4 M urea.

Fig. 2. Polyacrylamide gel electrophoresis of the salt-insoluble nonhistone proteins isolated from rat liver (1), rat mammary gland (2), and Walker tumor (3). Samples of approximately 200 µg of protein were used for each of the runs. Electrophoresis was from top (cathode) to bottom (anode) as described previously (18).

Chart 1. Template activities of chromatins of Walker tumor, rat liver, and mammary gland in RNA synthesis in vitro. Increasing amounts of chromatin, expressed in equivalent µg of DNA, as indicated, and 5.0 units of RNA polymerase were used in each reaction. The reaction mixture and assay were as described in "Materials and Methods."

chromatin exhibited less template activity than rat mammary and liver chromatins (Chart 1). Estimates from a double reciprocal plot of the data in Chart 1 showed that liver and mammary chromatins were 60 and 287%, respectively, more active than the tumor chromatin in promoting RNA synthesis. The chromatin template was restricted when compared to that of DNA, transcribing approximately 2, 5, and 10% of the sequences of DNA (Chart 2) of Walker tumor, liver, and mammary gland, respectively. Since bacterial polymerases transcribe as in vivo endogenous RNA polymerases do (23, 29, 32, 37), this result indicates a more suppressed genetic activity of the tumor chromatin than normal chromatins in directing RNA synthesis in vitro. Walker tumor cells are hyperdiploid.
and that some of these are trinucleated (35), and liver cells have been shown to contain diploid, tetraploid, and octaploid cells (6, 33). It has been suggested (46) that hyperploid portions of the genome may be those parts which are repressed. Such differences in ploidy may be reflected in template activity of the chromatin.

Hybridization of RNA's Transcribed from Normal and Tumor Chromatins. If the tumor chromatin represents a repression as well as derepression of normal gene activity, the additional genome sequences should be revealed by hybridization-competition experiments. Charts 3 and 4 show the results of annealing $^3$H-labeled RNA's transcribed from mammary gland and Walker tumor chromatins to mammary DNA. Hybridization of mammary RNA to mammary DNA gave a saturation plateau of 4.8%, while that of tumor RNA hybridized to mammary DNA was 2%, suggesting that different RNA's were being transcribed. This is further supported by the reciprocal double saturation hybridization data. The additional hybrid formed between tumor RNA and mammary DNA saturated with homologous RNA indicates new RNA species transcribed from tumor chromatin. Similar results were also obtained when the same RNA's were annealed to Walker tumor DNA (Charts 5 and 6).

Additional evidence showing that RNA transcribed from Walker tumor chromatin represents different RNA species than those transcribed from chromatins of normal tissues is supplied by the results obtained from hybridization of tumor RNA's transcribed from normal and tumor chromatins to Walker tumor DNA.
DISCUSSION

Our result showing the reduced template activity of Walker tumor chromatin on transcriptional level as compared with that of rat liver and mammary chromatins is in agreement with the finding of Omata and Ichii (28) and suggests a suppressed genetic activity in tumor chromatin. Busch (4) has reported that Walker tumor has decreased enzyme synthesis and activity as compared to normal tissues. Further, there is also a higher turnover and synthesis of histones in vitro (16, 19, 43, 44). These considerations thus suggest that the differences in chromosomal proteins may affect transcriptional activity in Walker tumor.

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REFERENCES

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