Changes in Hybridizable Nuclear RNA during the Neoplastic Development of Mouse Mammary Cells

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SUMMARY

The rapidly labeled nuclear RNA's in normal mammary glands, hyperplastic alveolar nodule (HAN) outgrowths, spontaneous mammary carcinomas, and serially transplanted mammary carcinomas of C3H mice were compared by the techniques of RNA-DNA hybridization and hybridization-competition. RNA derived from normal mammary gland competed incompletely with RNA-3 H from HAN outgrowths and still less completely with RNA-3 H from spontaneous carcinomas. RNA derived from HAN outgrowths competed incompletely with RNA-3 H from spontaneous carcinomas. RNA derived from spontaneous carcinomas competed incompletely with RNA-3 H from serially transplanted carcinomas. These results were verified in reverse labeling experiments. The progression of neoplastic development from normal cells to HAN's to spontaneous carcinoma to serially transplantable carcinoma cells is thus characterized by discreet increments at each stage in the diversity of the nuclear RNA species formed. The results suggest that altered regulation of transcription may be a feature of cells that have undergone the preneoplastic change, the neoplastic transformation, or neoplastic progression.

INTRODUCTION

Increasing evidence favors the viewpoint that the neoplastic transformation may be preceded by a progression through 1 or more preneoplastic stages and that cancer cells may continue to develop through multiple forms of the neoplastic state (12). In the mammary carcinoma system of the C3H mouse, adenocarcinomas develop from the preneoplastic HAN2 that arises from the glandular epithelium of older females (8, 10). Previous studies on a well-established form of this carcinoma, the C3HBA mammary adenocarcinoma, presented evidence for altered regulation of gene expression in these cells (27—29). Comparisons of rapidly labeled nuclear RNA populations by RNA-DNA hybridization-competition reactions demonstrated that these carcinoma cells form ribonucleotide sequences that are not detectable in the normal cells (29). In the present studies, nucleotide sequence homology studies have been performed to characterize alterations in gene transcription which may occur at discreet, intermediate stages in the transition from normal mammary cells to serially transplantable carcinoma cells. Specifically, RNA-DNA hybridization-competition reactions have been used to compare populations of rapidly labeled, hybridizable nuclear RNA from normal mammary gland, HAN outgrowths, small spontaneous carcinomas, and serially transplanted carcinomas. The results are considered in relation to a possible sequence of changes that may occur at the transcriptional level in association with neoplastic transformation and progression.

MATERIALS AND METHODS

Animals. Tissues derived from C3H/HeJ female mice were used in all experiments. The abdominal, inguinal, and thoracic mammary glands were removed from 4-month-old, 10-day lactating animals. Small, nonnecrotic spontaneous mammary adenocarcinomas were selected and pooled for study. C3HBA mammary adenocarcinomas were maintained by serial transplantation in C3H/HeJ female hosts. Primary HAN's were removed from the mammary glands of old, multiparous, nonpregnant, and nonlactating females and were transplanted into the "gland-free" inguinal mammary fat pad of 3-week-old females according to the transplantation technique of DeOme et al. (9). HAN outgrowths derived from the transplanted nodules were removed 12 weeks later.

Chemicals. Tritium-labeled nucleotides were purchased from Schwarz BioResearch, Orangeburg, N. Y., and inorganic phosphate32P was obtained from Tracerlab, Inc., Boston, Mass. Pancreatic RNase (5 times crystallized), T1 RNase (electrophoretically pure), and DNase (electrophoretically pure) were purchased from Worthington Biochemical Corp., Freehold, N. J.; Pronase was obtained from CalBiochem, Los Angeles, Calif.; subtilisin (Nagarse) was obtained from the Enzyme Development Corp., New York, N. Y.; RNase-free sucrose was a product of Mann Research Laboratories, Inc., New York, N. Y.; sodium dodecyl sulfate was a product of K and K Laboratories, Plainview, N. Y.; Millipore filters (25-mm diameter; type HAWP) were obtained from the Millipore Corp., Bedford, Mass.

Preparation of RNA. Minces of the normal and neoplastic mammary tissues were incubated at 37° under sterile conditions and were allowed to incorporate isotopic precursors for 30 min, as previously described (29). Highly purified nuclei were prepared from the tissue homogenates (30), and nuclear

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2The abbreviation used is: HAN, hyperplastic alveolar nodule.

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RNA was extracted and purified with phenol:m-cresol at 70° and a series of enzymatic digestion steps as previously described (29). All RNA samples used for hybridization were required to have a UV absorbance $A_{260} : A_{280}$ ratio of at least 1.85.

**Preparation of DNA.** DNA was extracted from mammary glands or liver by a modification of the method of Marmur (14). The DNA was further purified by incubation at 37° with pancreatic RNase (100 µg/ml) for 30 min, T$_1$ RNase (100 µg/ml) (31) for 20 min, and then Pronase (self-digested at 37° for 2 hr, 100 µg/ml) for 15 min. The incubation was terminated by the addition of 70% phenol, and the mixture was further deproteinized by 2 extractions with 70% phenol. Phenol was removed by repeated extractions with ether. The spooled DNA was dissolved in 2 times standard (0.15 M sodium chloride:0.015 M sodium citrate) and stored at 2° in the presence of chloroform, 2 µl/ml.

**RNA-DNA Hybridization Technique.** RNA-DNA hybridization reactions were carried out with a modification of the liquid-liquid annealing system of Nygaard and Hall (21, 22) as previously described (29). Heat-denatured DNA and RNA were incubated at 67° for 18 hr. The hybrid complexes were washed exhaustively, treated with pancreatic RNase, and assayed for radioactivity as previously described (29).

**RESULTS**

Saturation of DNA. The extent of RNA-DNA hybridization reached a plateau value after 14 hr of incubation at 67°. As shown in Chart 1, the amount of RNA-3H retained as a RNase-resistant hybrid was approximately proportional to the input up to an RNA:DNA ratio of 2. At higher ratios, relative saturation of available complementary binding sites on the DNA was approached. An RNA-3H:DNA ratio of 3 to 4 was usually adequate for relative saturation in the competitive hybridization reactions, but the “saturation” ratio was more precisely determined for each RNA preparation before these reactions were carried out. Chart 1 shows that the direct hybridization reaction did not detect differences in the RNA populations derived from HAN outgrowths or spontaneous carcinomas. These results are similar to those previously obtained with RNA preparations from mouse mammary gland and transplanted carcinomas (27).

**Comparison of RNA Populations by Hybridization-Competition.** Rapidly labeled nuclear RNA preparations from mammary gland, HAN outgrowths, spontaneous carcinomas, and transplanted carcinomas were compared in RNA-DNA hybridization-competition reactions. Chart 2 shows the results of a competition experiment in which unlabeled gland RNA and HAN RNA preparations were used in the hybridization reaction between HAN RNA-3H and homologous DNA. Competition by unlabeled HAN RNA approached theoretical competition values for identical populations of base sequences. RNA from the mammary gland competed less completely at all RNA:RNA-3H ratios tested. As a further test of the possible differences between these RNA's, a reverse labeling experiment was performed. HAN RNA was labeled with uridine-3H and adenine-3H. After the isotopic labeling period, both types of tissue minces were combined, and the RNA was purified and utilized for hybridization competition reactions under a double-label protocol. As shown in Chart 3, significant differences were observed. Mammary gland RNA competed incompletely with (HAN) RNA-32P in comparison to the relatively complete competition in the homologous hybridization-competition reaction. These results would appear to preclude the possibility that the differences observed were related to differences in precursor pool equilibrium or artifacts of the isolation procedure.

**Similar comparisons were made in**
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Chart 3. The competition of unlabeled mammary gland RNA in the hybridization reaction between DNA and mammary gland RNA-3H or HAN RNA-32P. The RNA's were labeled separately in vitro, isolated from a common homogenate, and counted by double-channel counting. Thirty µg of DNA were allowed to react with 110 µg of isotopically labeled RNA. Control hybridization values were 800 cpm for 3H and 1310 cpm for 32P.

hybridization-competition experiments in which RNA from HAN outgrowths and spontaneous carcinomas were utilized. Chart 4 shows that HAN RNA competed with HAN RNA-3H as essentially identical populations. However, a markedly incomplete degree of competition was evidenced by the HAN RNA preparation in the hybridization-competition reaction with RNA-3H from spontaneous carcinomas. In a competition reaction in which the isotopic label was reversed (Chart 5), a greater variety of hybridizable species of RNA in the spontaneous carcinoma was again observed in comparison with the HAN RNA. Significant differences between the HAN and gland RNA's were again observed in terms of their relative abilities to compete with a 3rd type of RNA-3H preparation, that from the spontaneous carcinoma.

Previous studies (29) demonstrated marked increases in the diversity of hybridizable nuclear RNA species in transplantable C3H mammary carcinomas in comparison with nonneoplastic mammary cells. Chart 5 also demonstrates that hybridizable nuclear RNA from mammary gland also competes incompletely with RNA-3H from spontaneous carcinomas. These differences were further substantiated by an experiment in which the isotopic label was reversed, as shown in Chart 6. Mammary gland RNA could not compete completely, as judged by the degree of competition in the homologous reaction, with RNA-3H from the spontaneous carcinomas.

Rapidly labeled, hybridizable nuclear RNA from spontaneous carcinomas was compared with similar preparations from transplantable carcinomas. As shown in Chart 7, experiments that utilized competition of unlabeled RNA's with RNA-3H from transplanted carcinomas revealed a crossing-over effect at low RNA:RNA-3H ratios. Effects observed at such low ratios are often difficult to interpret. However, at high levels of input RNA, significant differences in the 2 RNA preparations were consistently demonstrated. RNA from the spontaneous carcinomas competed incompletely with RNA-3H from the transplanted carcinomas.
Chart 6. The competition of unlabeled nuclear RNA from mammary gland in the hybridization reaction between DNA and mammary gland RNA-3H (96 μg; control hybridization, 907 cpm) or spontaneous carcinoma RNA-3H (85 μg; control hybridization, 1270 cpm).

Chart 7. The competition of unlabeled nuclear RNA from spontaneous or transplanted carcinomas in the hybridization reaction between DNA (36 μg) and RNA-3H (110 μg) from transplanted carcinoma. Control hybridization value was 1860 cpm.

Small but significant differences were consistently observed in the reverse label experiment (Chart 8), indicating the presence of RNA species in the transplantable carcinoma which were not detectable in the spontaneous carcinoma.

In the hybridization reactions shown in Chart 9A, RNA from mammary gland, HAN outgrowths, and spontaneous carcinomas were allowed to compete with mammary gland RNA-3H. All the hybridization curves approached theoretical values predicted for identical RNA populations competing with saturation-dependent kinetics. Similar experiments which compared the ability of transplanted carcinoma RNA to compete with RNA-3H from spontaneous carcinomas (Chart 9B) yielded competition curves which were not significantly different and which indicated complete competition.

An increasingly greater diversity of RNA populations was demonstrated in sequential comparisons of RNA from normal gland, HAN outgrowths, spontaneous carcinomas, and transplanted carcinomas. The results shown in Chart 9, A and B, indicate that this increasing diversity is not associated with any detectable loss of RNA species. However, the hybridization-competition technique is very insensitive for the detection of base sequence deletions (6).

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Since hybrid formation by mammalian nucleic acids does not require absolute complementarity of the entire ribonucleotide sequence; and since competition reactions
appear to assay primarily for redundant sequences (4), this failure to discriminate among the various subpopulations of RNA does not prove their identity. These results do, however, provide important control values. The significance of the differences in the competition experiments shown in Charts 2 to 8 may be evaluated in view of the relatively precise competition values obtained by these same techniques when the same RNA preparations are permitted to compete with mammary gland RNA-3H (Chart 9A).

DISCUSSION

A large body of evidence demonstrates that the mammary adenocarcinomas of C3H mice do not arise directly from normal mammary epithelial cells but arise instead from cell populations comprising HAN's. These HAN cells differ experimentally from normal mammary cells in terms of their altered hormonal responsiveness (3, 19), their apparently unlimited life-span in serial transplantations (7), and their ability to produce mammary neoplasms (10). The normal mammary gland, the HAN cells, and the spontaneous carcinomas to which the HAN cells give rise represent distinct cell types, since each of these types of mammary tissue maintains its identity throughout several serial transplantations into the gland-free mammary fat pads of isologous host mice (8, 9). The transformation of normal gland cells into the preneoplastic HAN lesion and its subsequent neoplastic transformation have been regarded as major and obligatory successive steps in the development of most mammary adenocarcinomas of C3H mice. Progression of carcinoma cells to the further acquisition of new, intrinsic characteristics is likely upon serial transplantation of the originally spontaneous carcinomas (2). Indeed, the presence of morphological (11, 23), biochemical (27, 28), and growth (16) characteristics which are distinct from those of the spontaneous carcinomas has been previously documented in the C3HBA transplanted carcinoma used in the present studies.

The present studies demonstrate that each of the successive stages of neoplastic development, preneoplastic nodular outgrowths, spontaneously transformed cells, and serially transplanted carcinoma cells, is characterized by a discreet increment in the diversity of hybridizable nuclear RNA species formed. Previous studies have demonstrated that the differentiation of mammary epithelial stem cells into secretory alveolar cells is associated with the formation of new hybridizable nuclear RNA species (24). Comparisons of secretory mammary tissue with serially transplanted carcinoma cells demonstrated a marked further increase in the variety of rapidly labeled, hybridizable RNA species present in these carcinoma cells. A more detailed analysis of intermediary stages between normal and transplantable neoplasms in the present experiments demonstrates progressive increases in the diversity of hybridizable RNA species. The rapidly labeled nuclear RNA from the HAN outgrowths exhibits a greater degree of competition than does RNA from the normal gland in hybridization-competition reactions with spontaneous carcinoma RNA. This result suggests that some of the differences between the gland and the spontaneous carcinoma RNA's may represent gene products, the synthesis of which is activated in the HAN cells and which continue to be formed in the neoplastic cells. Competition experiments with labeled gland RNA failed to detect any degree of incomplete competition by the HAN or carcinoma RNA. However, since the hybridization-competition reaction is insensitive to deletions, this result does not preclude the possibility that some areas of the genome transcribed in the gland or HAN outgrowths are no longer transcribed in the neoplastic cells. Although the hybridization technique provides information about qualitative changes in the populations of RNA's that are compared, it provides no information about the products of specific genes and gives no clue about the functions of the RNA molecules which are compared.

Previous studies on the nature of the rapidly labeled RNA of mouse mammary gland have indicated that it consists predominantly of 45 S and 32 S preribosomal RNA and of heterogeneous RNA with sedimentation constants ranging as high as 100 S and with a base composition which indicates that it is DNA-like RNA (26). Since the preribosomal RNA is homologous to less than 0.1% of the genome (1, 15, 18), it would appear likely that differences in the base sequence composition of the DNA-like RNA's account for the differences in the hybridization-competition reactions observed with rapidly labeled RNA in these experiments. Much of the DNA consists of reiterated sequences, and the conditions of these experiments permit detection of differences among only the redundant or partially redundant RNA sequences (4, 32). As such, the results are only a minimal estimate of actual differences which may exist in the RNA populations tested. At the present time the technique of RNA-DNA hybridization-competition appears to represent the most sensitive available one for the detection of sequence differences among RNA's. However, since the kinetics of RNA-DNA annealing in these reactions is largely unknown, the present results represent a preliminary attempt to characterize differences that may exist among the populations of RNA that have been tested.

The technique of RNA-DNA hybridization requires larger amounts of nucleic acids than can be extracted from a single, small carcinoma. For this reason, it was necessary to pool the glands, the HAN outgrowths, and the carcinomas for the extraction of their respective RNA's. This fact precludes the conclusion that the differences demonstrated are a requisite characteristic of all such tumors. The results represent an average value for a population of tumors. However, the differences observed were constant and reproducible with RNA preparations from a large number of carcinomas in a large number of experiments.

The formation of new, hybridizable RNA species has been shown to be a characteristic of cell differentiation in a number of systems (25). An increase in the diversity of hybridizable nuclear RNA's in comparison to the nonneoplastic cells has also been demonstrated in human chronic lymphocytic leukemia cells (20), in minimal deviation Morris hepatomas (17), and in Yoshida ascites hepatoma cells (5). The RNA transcripts from a given myeloma tumor have been distinguished from those of other myeloma tumors by RNA-DNA hybridization reactions (13). The acquisition of
neoplastic characteristics has been regarded as a possible extension of the ability of cells to differentiate (12), although the neoplastic transformation may preclude transitions to functional states characteristic of differentiated, nonneoplastic cells (27). The continuously enlarging diversity of hybridizable RNA species that appear during the normal differentiation and during the neoplastic transformation of mouse mammary cells may represent a type of change at the molecular level which is consistent with this viewpoint. Analysis of the factors that regulate the activation of gene transcription may thus provide further insights into the nature of the neoplastic state.

REFERENCES

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