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DIBUTYRYL CYCLIC AMP TREATMENT MIMICS OVARIECTOMY: NEW GENOMIC REGULATION IN MAMMARY TUMOR REGRESSION

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SUMMARY: The in vitro translated proteins from poly(A)RNAs differed when hormone-dependent mammary carcinomas were compared during their growth and regression. Within 6 hours post ovariectomy the concentration of one protein band increased and those of two protein bands decreased in the regressing as compared to the growing tumors. The translated protein patterns of the regressing tumors were identical whether regression was induced by ovariectomy or dibutyryl cyclic AMP treatment. The results suggest that mammary tumor growth is subject to genomic regulation and that the same new genetic event occurs in dibutyryl cyclic AMP- and ovariectomy-induced regression.

INTRODUCTION: Growth of hormone-dependent mammary carcinomas is arrested by either treatment with $N^6,O^{2'}$-dibutyryl cyclic adenosine 3',5'-monophosphate (dibutyryl cyclic AMP) or by ovariectomy (1-3). An increase in lysosomal hydrolytic enzyme activities was found to be one of the earliest signs of tumor regression following ovariectomy or dibutyryl cyclic AMP treatment (2-4) and the increased activity was due to an increased synthesis of the enzyme (3-6). It was also reported that the pattern of the leucine-labeled cytosol proteins differed between growing and regressing tumors (7). These translational and post-translational events were likely the results of regression rather than the cause of the regression process. In the work reported here we explored the possibility that the regression process of hormone-dependent tumors is regulated by a transcriptional event and that the regressions produced by hormone removal and dibutyryl cyclic AMP treatment are evoked by similar, if not identical, mechanism.

MATERIALS AND METHODS: Primary rat mammary carcinomas induced by 7,12-dimethylbenz(α)anthracene were used throughout this study. Regression was produced by either ovariectomy or dibutyryl cyclic AMP treatment (10 mg/200 g rat/day, s.c.) (2). Both growing and regressing tumors were excised at the appropriate time and were quickly frozen in liquid nitrogen until use. Total RNA from tumors was extracted as described

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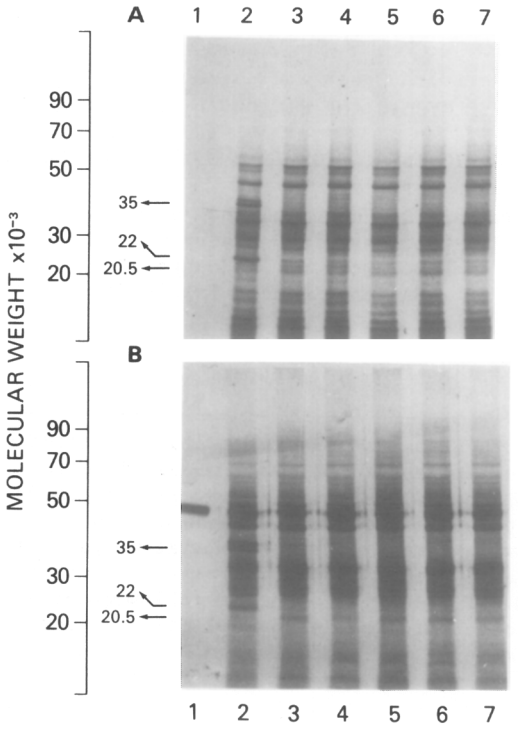

Fig. 1. Autoradiograms of the in vitro translation products of mRNAs from growing and regressing mammary tumors. (A) Translated in wheat germ extract S30 and (B) translated in rabbit reticulocyte lysate. Track 1 is the control where no mRNA was added; track 2 is with mRNA of growing tumors; tracks 3-7 are with poly (A)RNAs of ovariectomized regressing tumors: 3 = 6 hours; 4 = 16 hours; 5 = 36 hours; 6 = 3 days; 7 = 5 days after ovariectomy. Total RNA was extracted from 10 pooled tumors of each group as described by Deeley et al. (8) employing guanidine ·HCl. Poly(A)RNA was separated from total RNA with poly(U)-Sepharose affinity column and eluted with buffered formamide. Wheat germ extract was prepared according to the procedure of Roberts and Paterson (10) omitting the preincubation step. Protein synthesis was carried out in a final volume of 30 µl, containing 31.7 mM Hepes, pH 7.6, 100 mM KCl, 2.4 mM Mg·acetate, 1.8 mM DTT, 1.1 mM ATP, 0.2 mM GTP, 8.9 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 0.4 mM spermidine, 50 µM each of unlabeled amino acid, 10-20 µCi [^{35}S]methionine, 10 µl of wheat germ S30 extract and 0.8 µg poly(A)RNA. Reactions were incubated at 25°C for 90 min. Rabbit reticulocyte lysate was obtained from the Amersham Corporation, and the assays were carried out at 30°C for 90 min with 16 µl lysate, 2 µl [^{35}S] methionine (10-20 µCi) and 4 µl poly(A)RNA (0.8 µg). At the end of incubation, aliquots of 2 µl were removed and TCA-precipitable radioactivity was analyzed. The in vitro translation products in 5 µl of wheat germ system or 8 µl of reticulocyte system were denatured with 1 percent SDS and 1 percent β-mercaptoethanol and electrophoretically separated on a 10 percent SDS-polyacrylamide slab gel at 30 mA for 4 hours. At the end of the run the gel was soaked in a solution of 10 percent TCA, 10 percent acetic acid and 30 percent methanol, dried and exposed to Kodak X-Omat AR film for 12-36 hours. The data represent one of several experiments that gave essentially the same results.

by Deeley et al. (8). Poly(A)-containing RNA was isolated from the total RNA by poly(U)-Sepharose affinity column chromatography (9). Both wheat germ S30 extract and rabbit reticulocyte lysate were used to analyze the translatable activity of poly(A)RNA of growing and regressing tumors (10,11). The in vitro translation products were analyzed by SDS-polyacrylamide gel electrophoresis as described by Laemmli (12).

RESULTS AND DISCUSSION: Autoradiograms of [^{35}S]methionine-labeled translation products after the electrophoresis are presented in Fig. 1. As early as 6 hours after ovariectomy, two translation products with molecular weights of 22K and 35K (often appeared as a doublet) were significantly less in the regressing tumors than in the growing tumors. On the other hand, the regressing tumors had an increased amount of one translation product with a molecular weight of 20.5K. These differences were evident when the poly(A) RNAs from growing and regressing tumors were translated in either wheat germ extract or rabbit reticulocyte lysate. It is possible that various RNAs were translated with different efficiencies in the *in vitro* wheat germ or reticulocyte system. However, when comparisons are made within a single species of RNA, a different degree of translation would likely mean the presence of different levels of the message. Therefore, our data indicate that at least two species of mRNA are reduced and one is increased during regression. Despite the differences in transcription, the total amount of extractable RNA and poly(A)RNA as well as their translation activities per unit RNA were fairly constant up to 5 days after ovariectomy. After 5 days, both the amount of extractable RNA and its translatable activity declined. Rouleau and Gullino (13) also reported that during the first 3 days of tumor regression, despite a 30-50% reduction in tumor volume, there was very little reduction in the *in vivo* incorporation of labeled amino acid and uridine.

We next examined the translation products of poly(A)RNAs isolated from dibutyryl cyclic AMP-induced regressing tumors. As shown in Fig. 2, the three translation products differed again in their concentrations from those of growing tumors. Importantly, the same three protein species exhibiting either decreased or increased concentrations were found in regressing tumors after dibutyryl cyclic AMP treatment as were found in ovariectomy-induced regressing tumors (Fig. 2). Judging from the protein patterns of tumors which had regressed for 6 hours, the reduction of the two protein bands was more rapid with ovariectomy than that by dibutyryl cyclic AMP treatment.

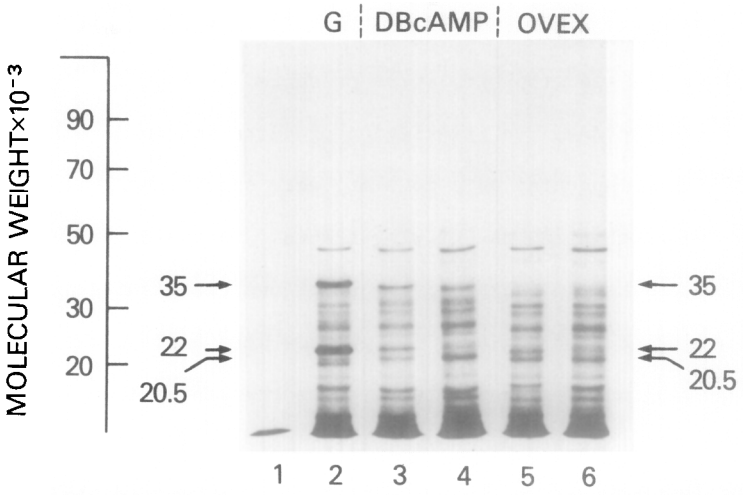

Fig. 2 Autoradiograms of in vitro translated products of mRNA from regressing tumors of dibutyryl cyclic AMP treated and ovariectomized rats. Track 1 is the control where no mRNA was added; track 2 is with RNA of growing tumors; tracks 3 and 4 are with RNAs of regressing tumors at 6 and 16 hours post dibutyryl cyclic AMP treatment, respectively; tracks 5 and 6 are with RNAs of regressing tumors at 6 and 16 hours post ovariectomy, respectively. The mRNAs were isolated from 10 pooled tumors of each group and translation was carried out in wheat germ extract S30 as described in Fig. 1. The data represent one of three experiments that gave essentially the same results.

Injections (2) of estradiol valerate into the ovariectomized host have produced resumption of tumor growth and reversed the changes in the translated protein pattern observed during regression (data not shown). Moreover, approximately 12% of DMBA-induced tumors that have failed to regress and continued to grow after ovariectomy or dibutyryl cyclic AMP treatment exhibited protein patterns appreciably different from that of hormone-dependent DMBA tumors, and the patterns did not change after ovariectomy or dibutyryl cyclic AMP treatment (data not shown). Thus, the changes in translation products demonstrable during regression appear to be specifically related to the hormone-dependence of mammary tumors.

Our study, as far as can be determined, presents the first evidence of differential gene expression during growth and regression of hormone-dependent mammary carcinoma. As early as 6 hours post ovariectomy or dibutyryl cyclic AMP treatment when there is no appreciable change in tumor size, the in vitro synthesis of several polypeptides instructed by poly(A)RNAs of tumors was either depressed or stimulated, indicating that these changes in gene expression are

an early event of tumor regression. The changing levels of translated proteins could be due to either the changing rate of transcription of their mRNAs or changes in the stabilization or degradation of the mRNAs. Our striking finding that both dibutyryl cyclic AMP treatment and ovariectomy induced the same changes in the translation products favors the possibility of the former: it is unlikely that vastly remote chemical components, such as estrogen and cyclic AMP could be involved in the stablization or degradation of the same message.

The results support our earlier proposal (14) that estrogen and cyclic AMP may counteract each other at the nuclear level in the growth control of hormone-dependent mammary tumors. We have shown previously (15, 16) that cyclic AMP-binding activity is inversely related to estrogen-binding activity in hormone-dependent mammary tumors during their growth and regression. Moreover, translocation of cytoplasmic cyclic AMP-binding protein and protein kinase into the nucleus, and new phosphorylation of a nuclear protein occur in the regressing tumors after either hormone-removal (ovariectomy) or dibutyryl cyclic AMP treatment (16, 17). It is probable that the antagonistic action between cyclic AMP and estrogen is responsible for the differential genetic expression observed in the mammary tumors. Studies on the isolation and identification of the specific mRNAs associated with the growth and regression of mammary tumors are currently under way.

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