Vol. 87, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 13, 1979 Pages 781-788

REGULATION OF MOUSE MAMMARY TUMOR VIRAL RNA SYNTHESIS IN EMBRYONAL CARCINOMA CELLS AND IN TERATOCARCINOMA DERIVED MYOBLASTS

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Received February 27,1979

SUMMARY

The expression of the mouse mammary tumor virus (MMTV) was studied in undifferentiated embryonal carcinoma cells (ECC), in partially differentiated myoblastic cells derived from ECC cells, and in fully differentiated myotubes. Whereas no appreciable amount of MMTV RNA could be detected in embryonal carcinoma cells, hybridation with radioactive viral cDNA revealed relatively large quantities of tumor virus RNA in the teratocarcinoma derived myoblasts. The MMTV RNA level was strongly reduced after differentiation of myoblasts into myotubes. The glucocorticoid hormone dexamethasone which stimulates the MMTV RNA synthesis in differentiated mammary cells did not affect this synthesis in myoblastic cells. By contrast, the apparently repressed synthesis of MMTV RNA in myotubes was almost completely overcomed with dexamethasone.

INTRODUCTION

Viral particles are often detected in embryonic and neonatal tissue but they do not seem to affect later development. Thus, murine leukemia virus particles readily multiply in mouse embryo without causing tumors and they act only in aged mice as a disease causing agent (1, 2). The fact that C type particles are so frequent during embryonic life and are not detectable in normal adult cells (3) might have some interesting albeit not fully appraised significance. Hence, it might be worth having a model in vitro system whereby to study the modulation of viral expression in the embryonic and in the differentiated stages.

Abbreviations used are : MMTV, mouse mammary tumor virus ; ECC, embryonal carcinoma cells ; PCC3, primitive carcinoma cells (clone 3) ; PCC4, primitive carcinoma cells (clone 4) ; TNE, Tris base, NaCl, EDTA ; SDS, sodium dodecyl sulfate ; Dex, dexamethasone.

0006-291X/79/070781-08$01.00/0

The mouse mammary tumor virus, MMTV, discovered by Bittner (4) in the milk of mice of the C_3H strain, is an endogenous virus closely related to the murine leukemia viruses. MMTV particles contain two molecules of 35 S RNA transcribed from proviral sequences found to be integrated in the genomes from all the mouse strains thus far studied (5). The expression of MMTV, generally as exogenously introduced DNA, is hormone dependent (6) in some strains, like C_3H or GR, and it has previously been shown that MMTV RNA synthesis in a mammary GR cell line is stimulated by the glucocorticoïd receptor protein complex (7, 8, 9). From the considerations outlined above, it was therefore of interest to analyze the control of MMTV expression during cell differentiation. In the present study we have compared the level of expression of this virus in some strains of murine embryonal carcinoma in tissue culture, as well as in some myoblastic lines derived from the same teratoma and cultivated either in the blastic stage or at the stage of early fusing myotubes. The sensitivity of MMTV RNA synthesis to glucocorticoïd hormones has also been examined.

MATERIAL AND METHODS

Cell culture and dexamethasone treatment. The embryonal carcinoma lines (PCC3, PCC4) and myoblast cell line C17-S1-D-T984 were previously described (10, 11). Cells were grown in Dulbecco's modified Eagle medium (12) containing 15% fœtal calf serum on 10 cm Falcon tissue culture dishes, in humidified incubators with 10% carbon dioxide 90% air. Embryonal carcinoma cells (PCC3, PCC4) and myoblast cells were harvested during exponential growth. Fused myoblasts were obtained after 6 days of myoblast cell culture. Dexamethasone was added at 5.10^{-7} M to the exponentially growing myoblast and differentiated myotubes for 6 hours.

Preparation of RNA and RNA/DNA hybridization. Cells were pelleted, washed at 4°C in phosphate buffered saline. Cells at a concentration of 5.10^6 cells/ml were resuspended in TNE buffer (0.01 M Tris base, pH 7.5 ; 0.01 M NaCl, 0.01 M EDTA, 0.1 mM diethylpyrocarbonate). Cell suspension was then treated with 500 µg/ml pronase and 0.5% (w/v) SDS for 2 hrs at 37°C. Nucleic acids were extracted twice with phenol saturated with TNE and precipitated with ethanol. Pellets of nucleic acids resuspended in 10 mM Tris HCl, pH 7.6 plus 10 mM $MgCl_2$ were treated with DNase for 2 hrs at room temperature. RNA was reextracted with phenol, precipitated with ethanol and resuspended in a small volume of TE (0.01 M Tris base, pH 7.6 ; 3 mM EDTA) to a concentration of about 3 mg/ml.

DNA complementary (cDNA) to the MMTV RNA genome was synthesized using the endogenous DNA polymerase activity of the virus purified from mouse milk as described elsewhere (9). The cDNA prepared

TABLE I

MMTV expression in the embryonal carcinoma, teratocarcinoma derived and mammary cell lines

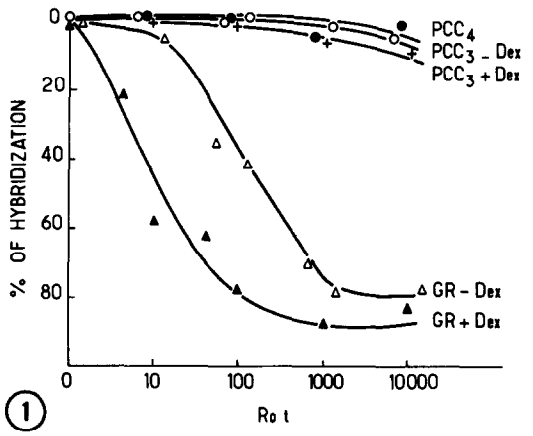
		MMTV RNA (copies/cell)	
		- Dex	+ Dex
Embryonal carcinoma	PCC3	0	0
" "	PCC4	0	nd
Myoblast	C17-S1-D-T984	1300	1300
Myotubes	C17-S1-D-T984	30	600
Mammary cells	GR	2000	23 000

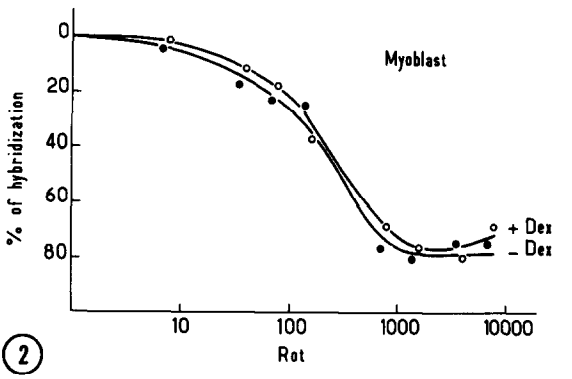
Conversion to number of copies/cell was established by assuming Rot 1/2 = 50 to be equivalent to 5500 copies/cell.

with (^{3}H) thymidine-5'- triphosphate (50 Ci/m mole) had specific activity of 10^8 cpm/ µg. The viral RNA concentration within RNA extracted from cells was determined by annealing increasing amounts of RNA with 1000 cpm of cDNA at 68°C in annealing buffer (0.6 M NaCl, 0.05 M Tris HCl, pH 7.4, 0.003 M EDTA). The amount of RNA:cDNA hybrid structure was determined by resistance to single strand specific S_1 nuclease.

RESULTS

Although MMTV specific nucleotide sequences are known to exist in multiple copies in all tested strains of laboratory mice (5), viral particles could be related to the expression of other endogenous viruses. A closer examination of the extent of MMTV expression in the various cell lines under study required that the concentration of MMTV RNA be measured by nucleic acid hybridization. The complementary DNA used as a probe to detect specific MMTV RNA sequences was made with the endogenous RNA directed DNA polymerase of MMTV particles released in the milk of mouse. The concentration of viral RNA in the cells (Table I) can be estimated from the initial RNA concentration (Ro) and the time (t) necessary for annealing the radioactive-cDNA. The ^{3}H-cDNA probe was hybridized to an excess of total RNA purified from the embryonal carcinoma strains (PCC3, PCC4) and GR mammary cells (Fig. 1), or from the myoblastic C17-S1-D-T984 cell line (Fig. 2, 3) taken





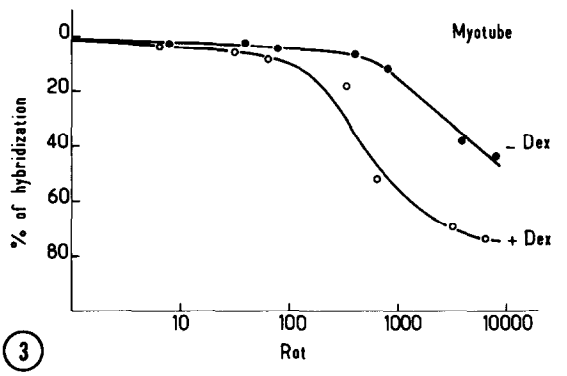


Fig. 1 : Hybridization with MMTV cDNA of RNAs from nullipotent PCC4 ECC(+), multipotent PCC3 ECC (o , ●) and GR mammary cells (▲ , Δ) . Culture cells were non treated (o, Δ) or treated (● , ▲) with dexamethasone for 6 hours (5 10^{-7}M).

Fig. 2 : Kinetics of hybridization of MMTV RNA from myoblastic cells non treated (—●—) or treated (—o—) with dexamethasone. Cells were treated with 5. 10^{-7}M dexamethasone for 6 hours. RNA was extracted and hybridized as described in "Material and Methods".

Fig. 3 : Hybridization with MMTV cDNA of RNA from fused myoblast non treated (—●—) or treated (—o—) with dexamethasone. Fused myoblastic cells were treated as undifferentiated myoblast. RNA was extracted and hybridized as described in "Material and Methods".

at various developmental stages, either before or after myotube formation. From the hybridization curves of Fig. 1, 2 and 3, the following can be concluded:

a) Undifferentiated ECC lines, whether multipotent like PCC3 or nullipotent like PCC4 do not contain appreciable amounts of MMTV RNA as compared to the GR epithelial mammary cell.

b) By contrast, a high concentration of MMTV RNA was detected in the myoblastic cell line derived from the undifferentiated embryonal carcinoma. Interestingly unfused myoblasts contain 20 fold more MMTV RNA than myotubes obtained from myoblasts grown for 6 days (Table I).

Experiments with dexamethasone (Dex), a synthetic glucocorticoid, have shown that mammary cells treated with this hormone for 6 hrs manufacture 13 fold more MMTV RNA than untreated cells (Fig. 1, Ref. 8, 9). Therefore, we have determined whether a similar treatment would influence MMTV RNA synthesis in unfused myoblasts and the derived myotubes. Whereas MMTV RNA synthesis in myoblastic cells proved unsensitive to dexamethasone, that occuring in myotubes was stimulated up to 20 fold. However the concentration of MMTV RNA in stimulated myotubes remained much lower than the concentration achieved in stimulated mammary cells (Table I).

DISCUSSION

This work clearly illustrates a correlation between the extent of cell differentiation in mouse cell lines and the expression of the endogenous MMTV virus. Whereas no MMTV RNA synthesis was shown to occur in embryonal carcinoma cells (PCC3, PCC4) important synthesis took place in C17-S1-D-T984 a myoblast line derived by subcloning from the same teratoma as PCC3. This synthesis of MMTV RNA was almost completely abolished after conversion of the mononucleate mouse myoblasts to multinucleate myotubes.

This differential expression of endogenous MMTV must be compared with results from studies on the response to virus infection of cells examined at various states of differentiation. It has already been observed that when they are infected with DNA viruses, like SV40 and polyoma, embryonal carcinoma cell lines show no evidence for viral expression (15, 16) whereas differentiated cell lines clearly support expression of these viruses. Moreover RNA viruses like mengovirus (17) or murine C type viruses (18, 19) do not replicate in embryonal carcinoma cells. In contrast they multiply very efficiently in differentiated derived cells (18, 19). For example, murine leukemia viruses readily infect and develop in myoblastic cell line (19). The resistance

of embryonal carcinoma cells to RNA viruses is not related to an early defect in the infection step (adsorption or penetration) but rather involves a later step (DNA integration or viral transcription) (19). Studies with SV40 virus suggest that the blockade in the expression of viral T antigen would be related to the process of early transcription or early messenger RNA "splicing".

Recent studies have shown that two transcripts of the MMTV genome contain sequences from the 5' terminus which are probably "spliced" during processing from a larger primary transcript (20).

Repression of MMTV expression in myotubes is at least partially overcome by treatment with the glucocorticoid hormone analog, dexamethasone. MMTV RNA synthesis in myotubes could be activated at the nuclear level by a dexamethasone receptor complex, although this remains to be proven. The high level of MMTV RNA found in myoblasts before fusion is not modified by dexamethasone treatment or at least not increased to the level characteristic of hormone inducible mammary cells. This lack of hormonal effect in myoblasts could be due either to the absence of cytoplasmic glucocorticoid receptor or to an inactive "chromatin site" for the receptor hormone complex.

An effect on the MMTV RNA stability is unlikely since it was shown by us (22) and others (13,14,23) that glucocorticoid hormones affect the initial rate of specific MMTV RNA production. To explain differences between myoblasts and myotubes we favour the hypothesis that two separate controls regulate the MMTV expression, one determining the baseline level and the other inducibility. Thus, MMTV genes experimentaly integrated into the genome of rat hepatoma cells (24), mink lung cells or cat kidney cells (25) are expressing at different levels and this expression is strongly stimulated by dexamethasone. On the other hand, like myoblasts, other non mammary mouse cells, such as the S 49 mouse lymphosarcoma cells, express MMTV genes at a high level in a hormone independent manner (7, 26). Inducible and non-inducible pathways of expression can be explained by assuming that the chromosomal locus at which an MMTV genome is located determines its phenotypic effects. In this respect, interesting insights would probably

be given by measuring the number of copies integrated in embryonal carcinoma cells, myoblasts and myotubes respectively.

Finally the ability of viral genes to respond in a larger spectrum of cultured cell types rules out the possibility that glucocorticoïd responsiveness is strictly limited to mammary cells. The process of glucocorticoïd activation could be analogous to positive control mechanisms during the induction of bacterial enzymes where binding at a specific promotor site triggers transcription of the adjacent gene. Acidic non-histone proteins which bind specifically to DNA could possibly be involved in this type of regulation.

ACKNOWLEDGEMENTS

We thank H. JAKOB who provided us with embryonal carcinoma cell cultures. We would like to acknowledge Prof. F. Jacob for helpful discussions and suggestions during the course of this work.

This wrok was supported by grants from Institut National de la Santé et de la Recherche Médicale, Délégation Générale à la Recherche Scientifique et Technique, Centre National de la Recherche Scientifique, Commissariat à l'Energie Atomique, Ligue Nationale Française contre le Cancer, Fondation pour la Recherche Médicale Française and Muscular Dystrophy Association.

REFERENCES

1. Dmochowski, L., Grey, C. E., Padgett, F. and Sykes, J. A. (1963) Viruses, Nucleic Acids and Cancer, p. 85, 17th Ann. Symp. Fund. Cancer Res., Williams et Wilkins, Baltimore, Md.
2. Feldman, D. G., Gross, L., and Dreyfus, Y. (1963) Cancer Res. 23, 1604-1626
3. Feldman, D. G. and Gross, L. (1966) Cancer Res., 26, 412-428
4. Bittner, J. J. (1942) Science, 95, 462-468
5. Varmus, H. E., Bishop, J. M., Nowinski, R. C. and Sarkar, N. H. (1972) Nature New Biol., 238, 189-191
6. McGrath, C. M., Nandi, S. and Young, L. J. T. (1972) J. Virol., 9, 367-374
7. Ringold, G. M., Yamamoto, K. R., Tomkins, G. M., Bishop, J. M. and Varmus, H. E. (1975) Cell, 6, 299-307
8. Lasfargues, E. Y. and Moore, D. H. (1972) Proc. Soc. Exp. Biol. Med., 139, 247-268
9. Ringold, G. M., Lasfargues, E. Y., Bishop, J. M. and Varmus, H. E. (1975) Virology, 65, 135-147
10. Jakob, H., Boon, T., Gaillard, J., Nicolas, J. F. and Jacob, F. (1973) Ann. Microbiol. (Inst. Pasteur) 124 B, 269-272

11. Jakob, H., Buckingham, M. E., Cohen, A., Dupont, L., Fiszman, M. and Jacob, F. (1978) Explt. Cell Res., 114, 403-408
12. Dulbecco, R. and Freeman, G. (1959) Virology, 8, 396-402

13. Ringold, G. M., Yamamoto, K. R., Bishop, J. M. and Varmus, H. E. (1977) Proc. Natl. Acad. Sci., 74, 2879-2889.
14. Young, H. A., Shih, T. Y., Scolnick, E. M. and Parks, W. P. (1977) J. Virol. 21, 139-146

15. Swartzendruber, D. E. and Lehman, J. M. (1975) J. Cell. Physiol. 85, 179-187
16. Kelly, F. and Boccara, M. (1976), Nature, 262, 409-410.
17. Lehman, J. M., Klein, I. B. and Hackenberg, R. M., in Teratomas and Differentiation (1975) eds. Sherman, M. I. and Solter, D., Academic Press p. 289-301
18. Teich, N. M., Weiss, R. A., Martin, G. R. and Lowry, D. R. (1977) Cell, 12, 973-982
19. Peries, J., Alves-Cardoso, E., Canivet, M., Debons-Guillemin, M. C. and Lasneret, J. J. (1977), Natl. Cancer Inst., 59, 463-465.
20. Varmus, H. E., personal communication
21. Aviv, H., Voloch, Z., Bastos, R. and Levy, S. (1977), Cell, 8, 495-503
22. Crépin, M. (1977) FEBS Letters, 84, 266-270
23. Yamamoto, K. R., Ivaric, R. D., Ring, J., Ringold, G. M. and Stallcup, M. R. (1978) Biochemical actions of hormones, ed. Litwack, G., in press
24. Ringold, G. M., Gardiff, R. D., Varmus, H. E. and Yamamoto, K. R. (1977) Cell, 10, 11-18.
25. Vaidya, A. B., Lasfargues, E. Y., Heubel, G., Lasfargues, J. C. and Moore, D. H. (1976) J. Virol., 18, 911-917.
26. Varmus, H. E., Quintrell, N., Medeiros, E., Bishop, J. M., Nowinski, R. C. and Sarkar, N. H. (1973) J. Mol. Biol., 79, 663-679.