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TRANSCRIPTION FACTOR LEVELS IN MEDULLARY THYROID CARCINOMA CELLS DIFFERENTIATED BY HARVEY RAS ONCOGENE: C-JUN IS INCREASED

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In the TT cell line of human medullary thyroid carcinoma, the viral Harvey ras ($v\text{-}ras^H$) oncogene induces differentiation, marked by morphological changes, diminution of growth, and increased expression of the calcitonin gene. Here, we show that the transcriptional factor c-jun is increased during $v\text{-}ras^H$ induced differentiation of TT cells both at the mRNA and functional protein levels. In contrast, nuclear proteins with binding activities related to AP2, AP3, NF1/CTF, and Sp1 were unchanged in $v\text{-}ras^H$ differentiated TT cells.

The ras family of oncogenes are best characterized for their transforming capabilities in rodent fibroblasts, and their activation in various experimental and naturally occurring cancers (1). However, in some cell types, ras oncogenes can play a role in cell differentiation. Introduction of ras genes or protein into PC12 rat pheochromocytoma cells induces neuronal differentiation accompanied by cessation of growth (2-4). We have noted in the TT cell line of human medullary thyroid carcinoma that introduction of $v\text{-}ras^H$ by infection with Harvey murine sarcoma virus (HaMSV) results in decreased growth, altered morphology, increased secretory granules, and increased expression of the calcitonin gene, including a change in the alternative splicing of the calcitonin gene transcript to more close resemble that of normal thyroid C-cells (5). Finally, Seremetis et al (6) have shown that introduction of $v\text{-}ras^H$ into human lymphoblastoid cells promoted plasmacytoid differentiation.

While these disparate effects of ras oncogene expression must involve signalling pathways leading to differences in gene expression, these pathways

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are not yet entirely defined. An important question is whether ras employs some of the same pathways in its role as a transforming agent or differentiating agent in different cells. In several genes which have been shown to be regulated by ras, the ras responsive DNA element contains an c-jun/AP1 binding site (7-11), suggesting that some of the effects of ras may be mediated through a jun family protein. Recently, it has been shown that in both 3T3 cells (12), in which ras is transforming, and in PC12 cells (13), in which ras is differentiating, the transcription factor c-jun is induced by ras. We have examined the modulation of several transcription factors in TT cells, in which ras has a differentiating function. Here, we show that c-jun is increased as a result of introduction of the v-ras^{H} gene into TT cells.

MATERIALS AND METHODS

Cell Culture. TT cells were grown in RPMI-1640 containing 16% fetal bovine serum, 100 U/ml penicillin, and 100 ug/ml streptomycin. They were infected with either HaMSV and amphotropic murine leukemia helper virus 1504A, or, as a control, with 1504A helper virus alone, as described previously (5).

Northern Blotting. Ten days postinfection, cells were harvested and RNA was isolated using guanidium isothiocyanate followed by oligo dT cellulose chromatography (14). Ten micrograms of poly A+ mRNA were blotted and hybridized, as previously described (5), with probes for c-jun/AP1 (15), NF1/CTF (16), or β-actin.

Mobility Shift Assay. Nuclear extracts were prepared from TT and TT ras cells by the method of Dignam et al. (17), except that buffer C contained 420 mM KCl instead of 420 mM NaCl. In addition, since TT ras cells apparently have high protease levels, leupeptin (0.5 ug/ml) and pepstatin (1 ug/ml) were added. Double stranded oligonucleotides containing a consensus binding site for AP1 (5'-CTAGTGATGAGTCAGCCGGATC-3'), AP2 (5'-GATCGAACTACCGCCCGCGGCCCGT-3'), AP3 (5'-CTAGTGGGACTTTCCACAGATC-3'), Sp1 (5'-GATCGATCGGGGCGGGGCGATC-3'), or NF1/CTF (5'-ATTTTGGCTTGAAGCCAATATG-3') were obtained from Stratagene and labeled to approximately 10^{8} cpm/ug by replacement synthesis using Klenow DNA polymerase and α-[^{32}P]-dCTP or α-[^{32}P]-dATP. Binding was done at 0^{o}C in 20 ul containing 2 x 10^{4} cpm oligonucleotide, 4.5-18 ug crude nuclear extract, 2 ug poly dI-dC, 50 mM KCl, 10 mM HEPES, 5 mM EDTA, 10% glycerol, pH 7.9. After 10-15 minutes, samples were loaded on a 5% polyacrylamide (79:1 acrylamide:bis) gel in 6.7 mM tris Cl, 3.35 mM Na acetate, 1 mM Na EDTA buffer, pH 7.9, and electrophoresed at 4^{o}C for 2 hours. Binding with each of these double stranded oligonucleotides could be blocked by excess cold homologous oligonucleotide (data not shown).

RESULTS

TT cells differentiated by infection by Ha-MSV (TT ras cells) express increased (5-15 fold) levels of c-jun mRNA, relative to c-jun levels in parental TT cells or TT cells infected by helper virus alone (Fig. 1A). In contrast, mRNA levels of the CCAAT box binding factor NF1/CTF were unchanged in TT ras cells (Fig. 1B). In support of this finding of increased c-jun mRNA in TT ras cells, we have also found an increase in functional jun family related

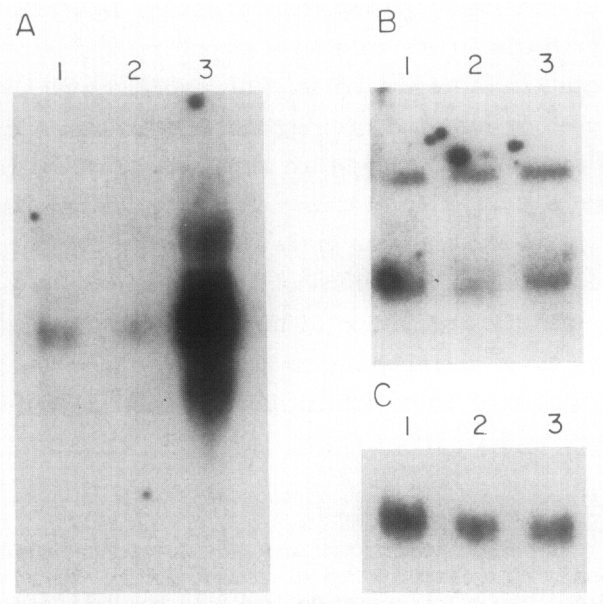

Figure 1. Increased levels of c-jun mRNA in TT cells differentiated by v-ras^{H} oncogene. Northern blot of poly A+ RNA from uninfected TT cells (lanes 1), TT cells infected with 1504A amphotropic murine leukemia helper virus (lane 2), and TT cells infected with Ha-MSV (lanes 3). A, c-jun mRNA detected by hybridization with pHJ (15); B, NF1/CTF detected by hybridization with pBS.CTF1 (16); C, β-actin mRNA for normalization of loading, detected by hybridization with a human β-actin probe, as described previously (5).

DNA binding activity in these cells. Nuclear extracts were prepared from TT and TT ras cells, and incubated with a double stranded oligonucleotide containing a consensus DNA sequence for the jun binding site. Binding activity for these oligonucleotides, which may be due to several members of the jun family, was detected by a gel mobility shift assay. As shown in Figure 2A, substantially more of the jun family consensus DNA binding site was bound when incubated with nuclear extracts from TT ras cells, suggesting that the c-jun mRNA is translated to functional protein.

Since ras induced differentiation of TT cells resulted in an increase in the c-jun transcription factor, we have used a similar gel mobility shift assay to examine whether this effect was specific, or whether numerous other transcription factors might generally be modulated by ras induced differentiation in these cells. We emphasize that this gel mobility shift assay does not identify the exact protein responsible for binding the oligonucleotide, but may detect one or more members of a family of DNA binding proteins with similar sequence specificity; the jun family, for example, has at least three members (18,19). We have noted no differences, in nuclear extracts from TT or TT ras cells, in levels of protein binding for oligonucleotides

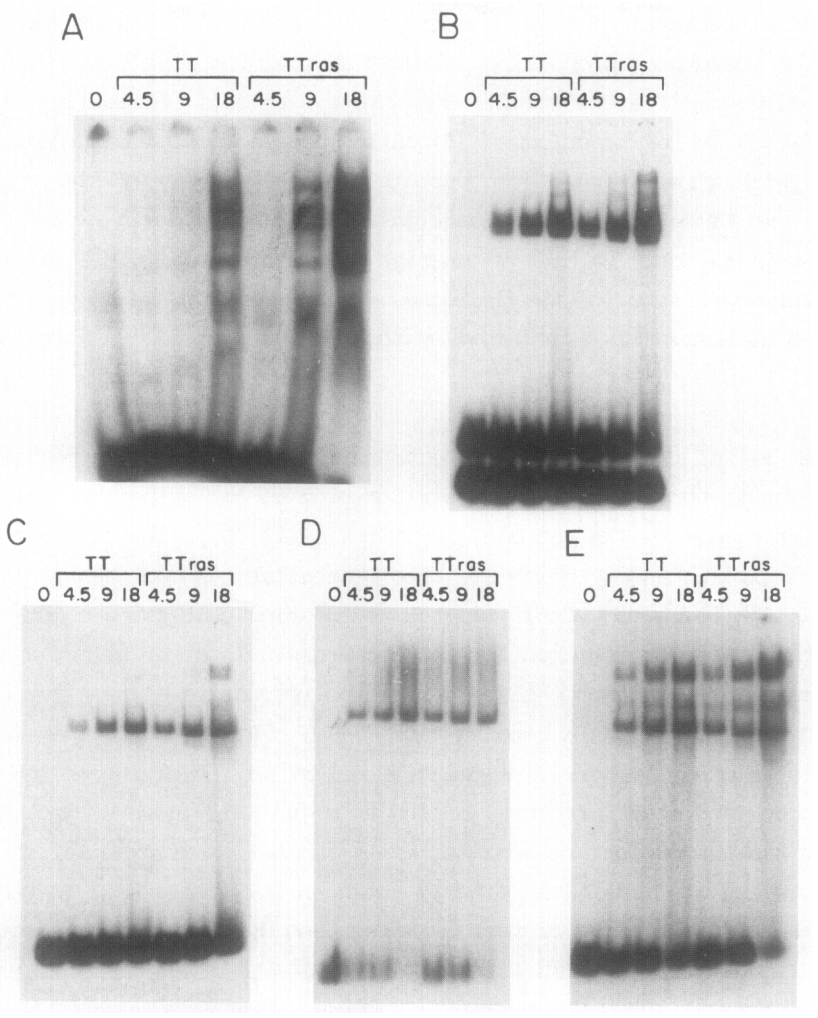

Figure 2. Increased levels of c-jun/AP1, but not AP2, AP3, NF1/CTF or Sp1 DNA sequence binding proteins in TT ras nuclear extracts. Labeled double stranded oligonucleotides containing the consensus sequences were incubated with indicated amounts (in micrograms) of nuclear extract from TT or TT ras cells, and protein-DNA complexes were separated from free oligonucleotides on nondenaturing 5% polyacrylamide gels, as described in Experimental Procedures. A, c-jun/AP1; B, AP2; C, AP3; D, NF1/CTF; E, Sp1.

containing the consensus sequence for transcription factors AP2 (20,21), AP3 (22), Sp1 (23) or NF1/CTF (24) (Fig. 2B-E), save for a possible increase in a minor high molecular weight AP3 band (Fig. 2C). The finding of no change in NF1/CTF binding is in agreement with our results showing no change in NF1/CTF mRNA in TT ras cells, and provides an internal control for possible differences in nuclear extract preparations.

DISCUSSION

In TT cells, as well as in 3T3 (12) and PC12 (13) cells, introduction of ras oncogenes results in increased expression of c-jun. This suggests that, at

least through the level of this transcriptional activator, ras may utilize identical or convergent signalling pathways whether the resultant phenotype is transformation or differentiation. Consistent with this possibility, it has been found that in ras-transformed 3T3 cells, phosphoinositide turnover is increased, with stimulation of the protein kinase C pathway (25,26). Similarily, in TT cells, phosphoinositide turnover and protein kinase C levels are increased (M. Mabry et al., manuscript in preparation). This pathway may be the upstream effector of the increases we have observed in c-jun levels, since c-jun expression is stimulated by this pathway in rodent fibroblasts (27,28).

Whether all of the phenotypic effects of ras in TT cells are mediated through c-jun is uncertain. In our earlier studies employing chemical differentiating agents TPA and cAMP on TT cells (29,30), some of the secretory and morphological changes, which are also seen after ras mediated differentiation, occurred within minutes, suggesting that changes in transcription were not required for those changes. In other cell lines, in several genes which are responsive to ras, the responsive element has been shown to contain a c-jun/AP1 binding site (7-11). By analogy, we envision that ras effects in other systems, including TT cells, may involve combinations of transcription (and other) factors, including c-jun/AP1. However, not all effects of ras expression are mediated via c-jun/AP1. For example, Sassone-Corsi et al. (13) have recently shown in PC12 cells that in the c-fos promoter the ras responsive element is within the dyad symmetry element and does not require the c-jun/AP1 binding site, although ras induced an AP1 like binding activity in those cells.

In TT cells, expression of the calcitonin gene is stimulated during v-ras^{H} mediated differentiation (5). The human calcitonin gene promoter contains the sequence TGACCTCA (31; B.D.N., unpublished) which Broad et al. (31) have noted is similar to the c-jun/AP1 consensus sequence. It will be important to determine whether this site is part of a ras responsive element, and whether this site is actually a sequence for c-jun/AP1 binding. In addition, the availability of cloned genes for some of these transcription factors, including c-jun/AP1, will allow dissection of their specific effects in differentiation or transformation.

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REFERENCES

1. Barbacid, M. (1987) Ann. Rev. Biochem. 56, 779-827.

2. Noda, M., Ko, M., Ogura, A., Liu, P.G., Amano, T., Takano, T., and Ikowa, Y. (1985) Nature 318, 73-75.

3. Bar-Sagi, D., and Feramisco, J.R. (1985) Cell 42, 841-848.

4. Hagag, N., Halegoua, S. and Viola, M. (1986) Nature 319, 680-682.

5. Nakagawa, T., Mabry, M., de Bustros, A., Ihle, J.N., Nelkin, B.D. and Baylin, S.B. (1987) Proc. Natl.. Acad. Sci. USA 84, 5923-5927.

6. Seremetis, S., Inghirani, G., Ferrero, D., Newcomb, E.W., Knowles, D.M., Dotto, G.P., and Dalla-Favera, R. (1989) Science 243, 660-663.

7. Imler, J.L., Schatz, C., Wasylyk, C., Chatton, B., and Wasylyk, B. (1988) Nature 332, 275-278.

8. Schonthal, A., Herrlich, P., Rahmsdorf, H.J., and Ponta, H. (1988) Cell 54, 325-334.

9. Offringa, R., Smits, A.M., Houweling, A., Bos, J.L., and van der Eb, A.J. (1988) Nuc. Acids Res. 16, 10973-10984.

10. Nunez, A.-M., Berry, M., Imler, J.L., and Chambon, P. (1989) EMBO J. 8, 823-829.

11. Yamaguchi, Y., Satake, M., and Ito, Y. (1989) J. Virol. 63, 1040-1048.

12. Sistonen, L., Holtta, E., Makela, T.P., Keski-Oja, J., and Alitalo, K. (1989) EMBO J. 8, 815-829.

13. Sassone-Corsi, P., Der, C.J., and Verma, I.M. (1989) Mol. Cell. Biol. 9, 3174-3183.

14. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.

15. Bohmann, D., Bos, T.J., Admon, A., Nishimura, T., Vogt, P.K., and Tjian, R. (1987) Science 238, 1386-1392.

16. Santoro, C., Mermod, N., Andrews, P.C., and Tjian, R. (1988) Nature 334, 218-224.

17. Dignam, J.D., Lebowitz, R.M., and Roeder, R.G. (1983) Nuc. Acids Res. 11, 1475-1479.

18. Nathans, D., Lau, L.F., Christy, B., Hartzell, S., Nakabeppu, Y., Ryder, K. (1988) Cold Spring Harbor Symposia on Quantitative Biology 53, 893-900.

19. Bravo, R., Zerial, M., Toschi, L., Schurmann, M., Muller, R., Hirai, S.I., Yaniv, M., Almendral, J.M., and Ryseck, R.P. (1988) Cold Spring Harbor Symposia on Quantitative Biology 53, 901-905.

20. Mitchell, P.J., Wang, C., and Tjian, R. (1987) Cell 50, 847-861.

21. Imagawa, M., Chiu, R., and Karin, M. (1987) Cell 51, 251-260.

22. Chiu, R., Imagawa, M., Imbra, R.J., Bockhoven, J.R., and Karin, M. (1987) Nature 329, 648-651.

23. Jones, K.A., Kadonaga, J.T., Rosenfeld, P.J., Kelly, T.J., and Tjian, R. (1987) Cell 48, 79-89.

24. Gidoni, D., Dynan, W.S., and Tjian, R. (1984) Nature 312, 409-413.

25. Fleischman, L.F., Chahwala, S.B., and Cantley, L.C. (1986) Science 231, 407-410.

26. Wolfman, A., and Macara, I. (1987) Nature 325, 359-361.

27. Angel, P., Hattori, K., Smeal, T., and Karin, M. (1988) Cell 55, 875-885.

28. Lamph, W.W., Wamsley, P., Sassone-Corsi, P., and Verma, I.M. (1988) Nature 334, 629-631.

29. de Bustros, A., Baylin, S.B., Berger, C.L., Roos, B.A., Leong, S.S., and Nelkin, B.D. (1985) J. Biol. Chem. 260, 98-104.

30. de Bustros, A., Baylin, S.B., Levin, M.A., and Nelkin, B.D. (1986) J. Biol. Chem. 261, 8036-8041.

31. Broad, P.M., Symes, A.J., Thakker, R.V., and Craig, R.K. (1989) Nuc. Acids Res. 17, 6999-7011.