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An Adenovirus 2-Coded Tumor Antigen Located on the Endoplasmic Reticulum and Nuclear Envelope Is Required for Growth of Transformed Cells in Ca^{2+}-Deficient Media

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Rat embryo cell lines containing the adenovirus 2 E1a region together with normal or mutant forms of the N-terminal half of the E1b region (*Hin*dIII G fragment) were generated by using a dominant selection marker, *neo*. Biochemically transformed cells containing a nonmutated *Hin*dIII G fragment proliferated more rapidly in Ca^{2+}-deficient media, whereas cells containing a specific deletion within the E1b-encoded, 175-amino-acid (175R) (19-kilodalton) T-antigen gene and nontransformed cells grew at a slower rate. Furthermore, transformed cells that did not express the 175R T antigen and untransformed cells could not replicate their DNA efficiently in low-Ca^{2+} medium. Our results suggest that Ca^{2+} ions may provide an important stimulus for cell proliferation in adenovirus-transformed cells through a mechanism that involves the functions of the 175R T antigen.

Experiments designed to identify the transforming genes of human adenoviruses by DNA transfection studies (12, 24) have shown that a DNA fragment from the leftmost 8% of the adenovirus 2 (Ad2) genome and the closely related Ad5 genome can fully transform rat cells in vitro. The leftmost 8% of the viral genome contains the entire E1a region (map position 1.2 to 4.3) and the N-terminal half of the E1b region. The normal E1b region contains the complete coding sequences for a T antigen of 175 amino acids (175R; 19 or 21 kilodaltons) and the N-terminal portion of the 495R T antigen (Fig. 1, bottom panel). DNA transfection studies have also shown that E1a-coding sequences contained within the leftmost 4.3% of the viral genome can only immortalize and partially transform rat cells (13). These results suggest that the N-terminal half of E1b is required for full transformation. Using a series of Ad2 mutants that produce large, clear plaques (*lp*) on human cells and have mutations within the 175R-coding region, we have provided genetic evidence that the 175R T antigen is important for cell transformation (5, 21). Similar results have also been obtained with Ad5 (1, 16, 22) and Ad12 (11, 14, 23). In addition to in vitro cell transformation, the 175R protein also appears to be required for tumorigenesis of Ad12-transformed cells in nude mice (2) and of Ad12 in hamsters (11, 23).

Cell fractionation and immunofluorescence studies have localized the 175R T antigen on the cytoplasmic membranes of transformed and productively infected cells (15, 18). More recently, this protein was localized predominantly on the endoplasmic reticulum and nuclear envelope by electron microscopy and immunoperoxidase staining (25). However, the biochemical function of this protein and, consequently, the precise mechanism of 175R T-antigen-mediated cell transformation and tumorigenesis is not known.

During transformation studies with an Ad2 mutant, *dl*250 (21), that is almost entirely defective in the 175R protein, we observed that this mutant transformed rat cells at greatly reduced frequencies, as compared with wild-type Ad2. However, the rarely occurring *dl*250-transformed cells appeared to be only partially transformed and, furthermore, did not grow well in Ca^{2+}-deficient media (results not shown). This observation contrasts with the well-established observation that adenovirus-transformed cells grow readily in media containing low amounts of Ca^{2+} ions (10). Our preliminary observation that *dl*250-transformed cells were defective in growth in Ca^{2+}-deficient media suggested to us that the 175R T antigen may be required for the growth of adenovirus-transformed cells. Since *dl*250-transformed cells contain other regions of the viral genome in addition to the E1a and E1b regions, we constructed rat embryo cell lines that contained only the leftmost 8% of the viral genome and specifically show in this report that the 175R T antigen is required for the growth of adenovirus-transformed cells in Ca^{2+}-deficient media.

An established rat embryo cell line, CREF, now commonly used for transformation studies (9), was transfected with plasmid pGC212, which contains the leftmost 8% of the viral genome (*Hin*dIII G fragment) or a mutant of pGC212 lacking the 175R protein-coding region (p*dl*250). Plasmid p*dl*250 contains a deletion of 145 base pairs between nucleotide positions 1768 (*Sst*I site) and 1912 (*Bst*EII site). Cells containing pGC212 or p*dl*250 DNA were selected by cotransfection with plasmid (ptk-neo) DNA coding for a dominant selectable marker, *neo* (6, 19). The G418-resistant (Neo^r) colonies were selected and screened for the presence of viral DNA. Two cell lines containing the nonmutated E1 region (i.e., the *Hin*dIII G fragment) and two cell lines containing the E1 region with a deletion of the 175R protein-coding region were selected from the preliminary screening. The viral DNA present in these cell lines was analyzed by Southern blot analysis (Fig. 1). Cell lines G1 and G3 contained about 5 and 10 copies of the *Hin*dIII G fragment, respectively, and cell lines *dl*2 and *dl*3 contained about 10 and 2 copies of the mutant form of the *Hin*dIII G fragment, respectively. The blot of DNA digested with restriction endonuclease *Pvu*II indicated that cell lines G1 and G3 contained the expected 1,874-base-pair fragment which encompasses most of the E1a-E1b-coding sequences within the *Hin*dIII G fragment. Cell lines *dl*2 and *dl*3 contained the expected 1,729-base-pair fragment. These results indicated

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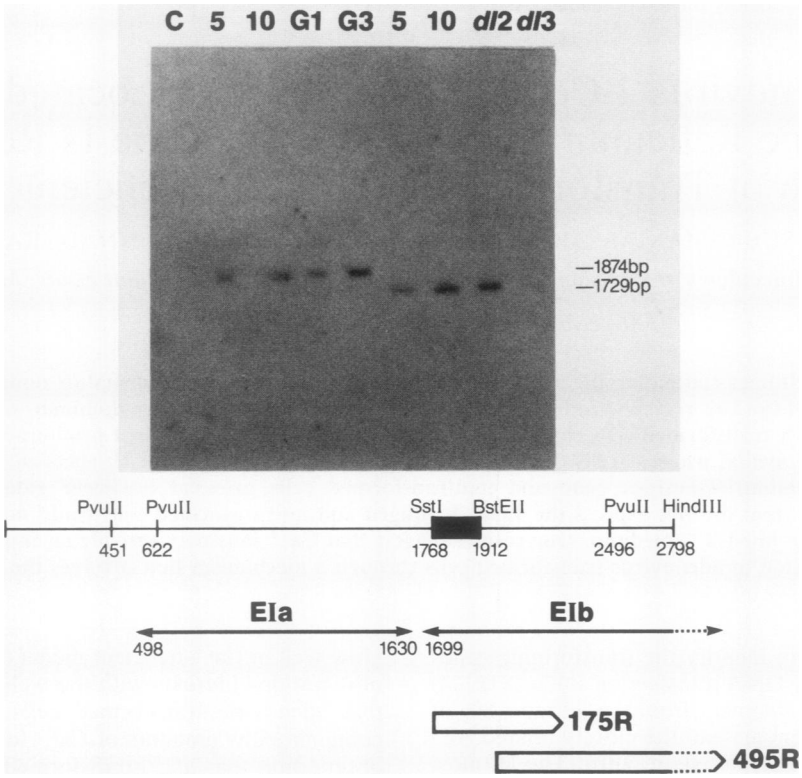

FIG. 1. Southern blot analysis of viral DNA present in transformed cells. CREF cells containing Ad2 DNA were selected by cotransfection with 9 μg of linearized (with *Eco*RI) plasmid DNA (pGC212 or p*dl*250) and 1 μg of plasmid ptk-neo DNA (constructed by T. H. Manoharan). Neo^r colonies were selected by using the antibiotic G418 (400 μg/ml). Cell DNA was cleaved with restriction endonuclease *Pvu*II, fractionated on a 1% agarose gel, denatured, immobilized on a Nytran membrane (Schleicher & Schuell, Inc.), and hybridized with the nick-translated *Hin*dIII G fragment (isolated from plasmid pGC212) in accordance with the protocol of the manufacturer. C, CREF cells; 5 and 10, copies of the plasmid DNA. bp, Base pairs.

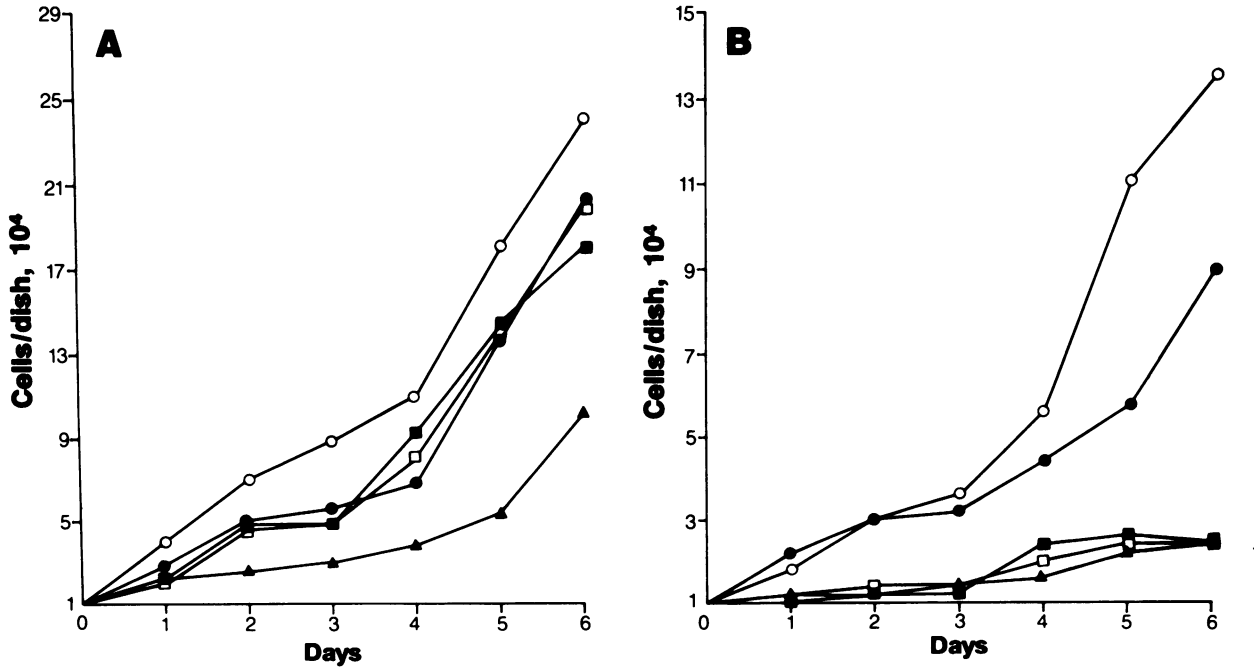

FIG. 2. Growth of transformed cells in normal (A) and Ca^{2+}-deficient (B) media. Cells were maintained in Ca^{2+}-deficient media for 48 h. About 10^4 cells were then seeded in 60-mm dishes, and at various times, cells were collected by trypsinization and counted. Symbols: ▲, CREF cells; ○, cell line G1; ●, cell line G3; □, cell line *dl*2; ■, cell line *dl*3.

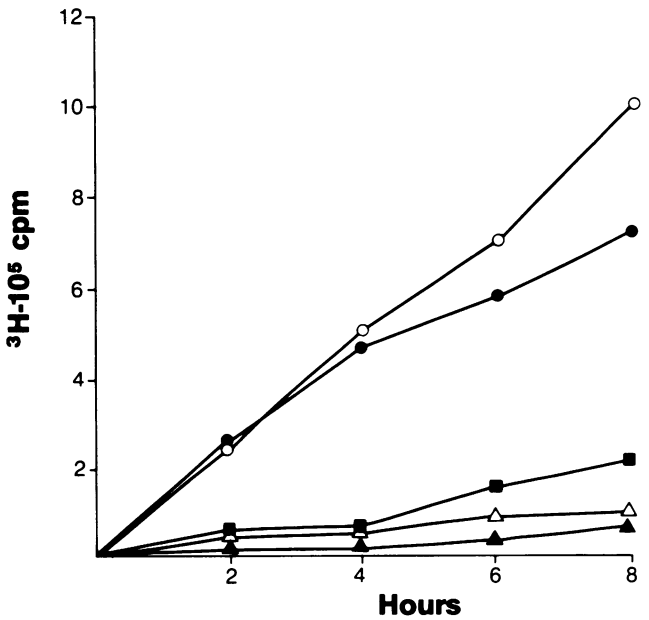

Fig. 3. DNA synthesis by transformed cells in Ca^{2+}-deficient media. Cells were maintained in Ca^{2+}-deficient media for 48 h and then trypsinized, and 10^6 cells were plated in Ca^{2+}-deficient media. After 24 h, cells were labeled with 50 μCi of [^{3}H]thymidine per ml (specific activity, 20 Ci/mmol). Labeled cells were collected by trypsinization, and radioactivity was determined after trichloroacetic acid precipitation. Symbols: ▲, CREF cells; ○, cell line G1; ●, cell line G3; ■, cell line *dl*2; △, cell line *dl*3.

that the E1a-E1b-coding regions do not have any major rearrangements in the transformed cells.

The growth properties of the various cell lines in normal media (1.8 mM Ca^{2+}) and Ca^{2+}-deficient media (no added Ca^{2+} other than the ions present in the given lot of dialyzed sera) were then compared. All the transformed cell lines containing viral DNA proliferated more rapidly than did the untransformed CREF cells (Fig. 2A). Among the transformed cell lines, cell line G1 grew at a slightly faster rate; no major difference was observed between the other three cell lines. In contrast to growth in normal media, in Ca^{2+}-deficient media, cell lines *dl*2 and *dl*3, which lack most of the 175R-coding sequences, grew like untransformed CREF cells and at a considerably slower rate than did both the G1 and G3 cell lines, which express the 175R T antigen (Fig. 2B). We have also observed similar growth defects in other cell lines transformed by viral mutant *dl*250 (20) in Ca^{2+}-deficient media (data not shown).

As a measure of cell proliferation, we also measured DNA synthesis by various cell lines in Ca^{2+}-deficient media (Fig. 3). Cells were starved for Ca^{2+} for about 72 h, and the incorporation of [^{3}H]thymidine into cellular DNA was measured during an 8-h labeling period. The results in Fig. 3 clearly show that cell lines *dl*2 and *dl*3 did not synthesize significant amounts of DNA, as compared with untransformed CREF cells, whereas cell lines G1 and G3 synthesized substantial amounts of DNA.

The results presented in this communication indicate that the 175R T antigen is important for cell proliferation in Ca^{2+}-deficient media. Ca^{2+} ions have been shown to provide an important stimulus to the onset of cell proliferation (3, 4, 7). The 175R T antigen may therefore be involved in Ca^{2+} mobilization in transformed cells. It is interesting to note that significant amounts of the 175R T antigen are located on the endoplasmic reticulum and nuclear envelope (25). Recent studies indicate that the endoplasmic reticulum is an important intracellular store of Ca^{2+} (17, 20), and the 175R protein may be involved in the uptake or release of Ca^{2+} by the endoplasmic reticulum. It is noteworthy that cells transformed by temperature-sensitive, transformation-defective mutants of avian sarcoma virus also exhibit similar growth defects in Ca^{2+}-deficient media (8). However, it is likely that the mechanisms of $pp60^{src}$ which possess tyrosine-specific protein kinase activity and 175R T-antigen-mediated cell proliferation may be different.

Although our results indicate that the 175R T antigen is required for the growth of adenovirus-transformed cells in Ca^{2+}-deficient media, possible synergistic roles of other virus-encoded proteins from the E1a region and the N-terminal region of the E1b 495R T antigen remain to be investigated.

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LITERATURE CITED

1. **Babiss, L. E., P. B. Fisher, and H. S. Ginsberg.** 1984. Effect on transformation of mutations in the early region 1B-encoded 21- and 55-kilodalton proteins of adenovirus 5. J. Virol. **52**:389–395.
2. **Bernards, R., P. I. Schrier, J. L. Bos, and A. J. Van der Eb.** 1983. Role of adenovirus type 5 and 12 early region 1B tumor antigens in oncogenic transformation. Virology **127**:345–360.
3. **Berridge, M. J.** 1984. Inositol trisphosphate and diacylglycerol as second messengers. Biochem. J. **220**:345–360.
4. **Berridge, M. J., and R. F. Irvine.** 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature (London) **312**:315–321.
5. **Chinnadurai, G.** 1983. Adenovirus 2 lp^+ locus codes for a 19-kd tumor antigen that plays an essential role in cell transformation. Cell. **33**:759–766.
6. **Colbère-Garapin, F., F. Horodniceanu, P. Kourilsky, and A. C. Garapin.** 1981. A new dominant hybrid selective marker for higher eukaryotic cells. J. Mol. Biol. **150**:1–14.
7. **Durham, A. C., and J. M. Walton.** 1982. Calcium ions and the control of proliferation in normal and cancer cells. Biosci. Rep. **2**:15–30.
8. **Durkin, J. P., A. L. Boynton, and J. F. Whitfield.** 1981. The *src* gene product ($pp60^{src}$) of avian sarcoma virus rapidly induces DNA synthesis and proliferation of deprived rat cells. Biochem. Biophys. Res. Commun. **103**:233–239.
9. **Fisher, P. B., L. E. Babiss, J. B. Weinstein, and H. S. Ginsberg.** 1982. Analysis of type 5 adenovirus transformation with a cloned rat embryo cell line (CREF). Proc. Natl. Acad. Sci. USA **79**:3527–3531.
10. **Freeman, A. E., S. Hollinger, P. J. Price, and C. Calisher.** 1965. The effect of calcium on cell lines derived from adenovirus type 12-induced hamster tumors. Exp. Cell Res. **39**:259–264.
11. **Fukui, Y., I. Saito, K. Shiroki, and H. Shimojo.** 1984. Isolation of transformation-defective, replication-nondefective early region 1B mutants of adenovirus 12. J. Virol. **49**:154–161.
12. **Graham, F. L., A. J. Van der Eb, and H. L. Heijneker.** 1974. Size and location of the transforming region in human adenovirus DNA. Nature (London) **251**:687–691.
13. **Houweling, A., P. J. Van der Elsen, and A. J. Van der Eb.** 1980. Partial transformation of primary rat cells by the left most 4.5% fragment of adenovirus 5 DNA. Virology **105**:537–550.
14. **Mak, I., and S. Mak.** 1983. Transformation of rat cells by *cyt* mutants of adenovirus type 12. J. Virol. **45**:1107–1117.
15. **Persson, H., M. G. Katze, and L. Philipson.** 1982. Purification of a native membrane-associated adenovirus tumor antigen. J. Virol. **42**:905–917.
16. **Pilder, S., J. Logan, and T. Shenk.** 1984. Deletion of the gene encoding the adenovirus 5 early region 1B 21,000-molecular-weight polypeptide leads to degradation of viral and host cell

DNA. J. Virol. **52**:664–671.
17. **Prentki, M., T. J. Biden, D. Janjic, R. F. Irvine, M. J. Berridge, and C. B. Wollheim.** 1984. Rapid mobilization of Ca^{++} from rat insolinoma microsomes by inositol-1,4,5-trisphosphate. Nature (London) **307**:562–564.
18. **Rowe, D. T., and F. L. Graham.** 1983. Transformation of rodent cells by DNA extracted from transformation-defective adenovirus mutants. J. Virol. **46**:1039–1044.
19. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
20. **Streb, H., R. F. Irvine, M. J. Berridge, and A. Schulz.** 1983. Release of Ca^{++} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature (London) **306**:67–68.
21. **Subramanian, T., M. Kuppuswamy, S. Mak, and G. Chinnadurai.** 1984. Adenovirus cyt^+ locus, which controls cell transformation and tumorigenicity, is an allele of lp^+ locus, which codes for a 19-kilodalton tumor antigen. J. Virol. **52**:336–343.
22. **Takemori, N., C. Cladaras, B. Bhat, A. J. Conley, and W. S. M. Wold.** 1984. *cyt* gene of adenovirus 2 and 5 is an oncogene for transforming function in early region E1B and encodes the E1B 19,000-molecular-weight polypeptide. J. Virol. **52**:793–805.
23. **Takemori, N., J. L. Riggs, and C. D. Aldrich.** 1968. Genetic studies with tumorigenic adenovirus. I. Isolation of cytocidal (*cyt*) mutants of adenovirus type 12. Virology **36**:575–586.
24. **Van der Eb, A. J., C. Mulder, F. L. Graham, and A. Houweling.** 1977. Transformation with specific fragments of adenovirus DNAs. I. Isolation of specific fragments with transforming activity of adenovirus 2 and 5 DNA. Gene **2**:115–132.
25. **White, E., S. H. Bloge, and B. W. Stillman.** 1984. Nuclear envelope localization of an adenovirus tumor antigen that maintains the integrity of cellular DNA. Mol. Cell. Biol. **4**:2865–2875.