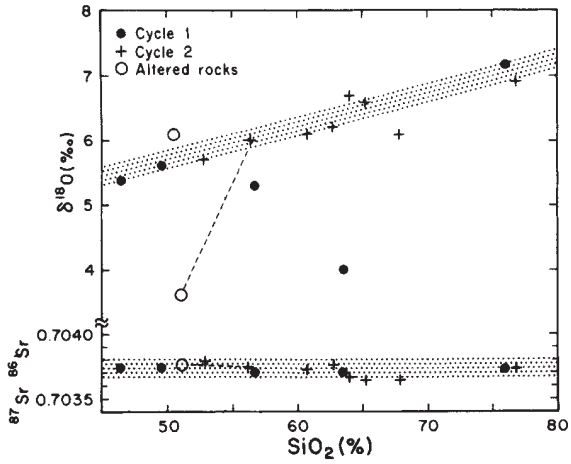

Fig. 5 Variation in whole-rock $\delta^{18}O$ values and initial $^{87}Sr/^{86}Sr$ ratios with % SiO_2 for samples from the Koloula Igneous Complex. Petrographic observations were used to characterize alteration. Tielines join altered and unaltered samples from the same intrusive phase.

Interpretation

The Koloula Igneous Complex is composed of 26 intrusive phases that range from gabbro to aplite. The youthfulness of the complex, its excellent exposure and our knowledge of its tectonic origins make the Koloula study unique in the geological literature. The geochemical variations seen within the two cycles of igneous intrusion are considered to be due to crystal fractionation. Within each cycle K/Rb and K/Ba ratios are similar and suggest that the members of each cycle are comagmatic. The difference in K/Rb and K/Ba ratios between cycles, with no difference in $^{87}Sr/^{86}Sr$ ratios and $\delta^{18}O$ values suggests a similar source for the magmas but different degrees of partial melting.

The large range in composition and relatively low and constant $^{87}Sr/^{86}Sr$ ratios observed in these rocks also makes it possible to model variations in oxygen isotope composition as related to crystal fractionation. Although such variations have been noted in volcanic rocks from Hachijo-Jima in the Izu–Mariana group[21], our data are the first to demonstrate these isotopic systematics in plutonic rocks resulting purely from crystal fractionation of a mantle-derived magma in a closed system. From our data which display relatively low $\delta^{18}O$ at high SiO_2 contents it becomes increasingly evident that examples of the more potassic suite, gabbro → granodiorite → granite are not a result of closed-system differentiation in either small (for example Ploumanac'h[30]) or large plutonic bodies (for example, Peninsular Ranges[31]). The evolution of these granites involves magma contamination by continental crust or partial melting of mixed mantle/continental source regions. Volcanic rocks from the Banda Arc also exhibit a correlation of $^{86}Sr/^{86}Sr$ with $\delta^{18}O$ that has been interpreted as being the result of contamination by crustal material or by subducted sediments[32].

From the comparisons with the limited data available from other young island-arc plutonic complexes, it appears that in unaltered complexes distinctions that are based on trace-element chemistries and isotopic ratios may be made between oceanic and continental environments.

Before this can be applied rigorously to plutonic complexes of uncertain tectonic environment, we need many more detailed studies of young complexes in known tectonic terrains. When this data base is established it may be then applied to older orogens where plutonic rocks are commonly the only remnants of volcanic activity.

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1. Jakeš, P. & White, A. J. R. *Bull. geol. Soc. Am.* **83**, 29–40 (1972).
2. Ewart, A., Brothers, R. N. & Mateen, A. *J. Volcanol. Geotherm. Res.* **2**, 205–250 (1977).
3. Kroenke, L. W. *Hawaii Inst. Geophys., Univ. Hawaii Rep.* HIG-72-5 (1972).
4. Hackman, B. D. thesis, Univ. Western Australia (1971).
5. Furumoto, A. S. *et al. Pacif. Sci.* **24**, 315–332 (1970).
6. Chivas, A. R. *Bull. Aust. Soc. Explor. Geophys.* **6**, 64–65 (1975).
7. Chivas, A. R. *Econ. Geol.* **73**, 645–677 (1978).
8. Chivas, A. R. & McDougall, I. *Econ. Geol.* **73**, 678–689 (1978).
9. Chivas, A. R. & Wilkins, R. W. T. *Econ. Geol.* **72**, 153–169 (1977).
10. Chivas, A. R. *Contr. Miner. Petrol.* **78**, 389–403 (1981).
11. Hendry, D. A. F., Chivas, A. R., Reed, S. J. B. & Long, J. V. P. *Contr. Miner. Petrol.* **78**, 404–412 (1981).
12. Chivas, A. R. thesis, Univ. Sydney (1977).
13. Taubeneck, W. H. *J. geol. Soc. Am. Spec. Pap.* **91**, 1–56 (1967).
14. White, A. J. R. & Chappell, B. W. *Tectonophysics* **43**, 7–22 (1977).
15. Taylor, H. P. Jr *Econ. Geol.* **69**, 843–883 (1974).
16. Faure, G. & Powell, J. L. *Strontium Isotope Geology* (Springer New York, 1972).
17. McCulloch, M. T., Gregory, R. T., Wasserburg, G. J. & Taylor, H. P. Jr *Earth planet. Sci. Lett.* **46**, 201–211 (1980).
18. O'Neil, J. R. & Taylor, H. P. Jr *Am. Miner.* **52**, 1414–1437 (1967).
19. Matsuhisa, Y. *J. Volcanol. Geotherm. Res.* **5**, 271–296 (1979).
20. Takita, R. *J. geol. Soc. Jap.* **80**, 505–523 (1974).
21. Ishizaka, K. & Yangi, T. *Earth planet. Sci. Lett.* **33**, 345–352 (1977).
22. Gill, J. B. *Contr. Miner. Petrol.* **27**, 179–203 (1970).
23. Ewart, A., Bryan, W. & Gill, J. *J. Petrol.* **14**, 429–465 (1973).
24. Perfit, M. R., Brueckner, H., Lawrence, J. R. & Kay, R. W. *Contr. Miner. Petrol.* **73**, 69–87 (1980).
25. Marsh, B. D. in *Andesites: Orogenic andesites and related rocks* (Thorpe, R. S.) (Wiley, New York, 1982).
26. Lopez-Escobar, L., Frey, F. A. & Oyarzun, J. *Contr. Miner. Petrol.* **70**, 439–450 (1979).
27. Cater, F. W. *U.S. geol. Surv. Spec. Pap.* **116**, 1–54 (1969).
28. Fiske, R. S., Hopson, C. A. & Waters, A. C. *U.S. geol. Surv. Prof. Pap.* **444**, 1–93 (1963).
29. Meijer, A. thesis, Univ. California, Santa Barbara (1971).
30. Albarède, F., Dupuis, C. & Taylor, H. P. Jr. *J. geol. Soc. Lond.* **137**, 641–647 (1980).
31. Silver, L. T., Taylor, H. P. Jr & Chappell, B. W. *Geol. Soc. Am. A. Meet. Field Trip Guide* 83–110 (San Diego State University, California, 1979).
32. Magaritz, M., Whitford, D. J. & James, D. E. *Earth planet. Sci. Lett.* **40**, 220–230 (1978).

Mechanism of activation of a human oncogene

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The oncogene of the human EJ bladder carcinoma cell lines arose via alteration of a cellular proto-oncogene. Experiments are presented that localize the genetic lesion that led to activation of the oncogene. The lesion has no affect on levels of expression of the oncogene. Instead, it affects the structure of the oncogene-encoded protein.

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A LARGE body of work on chemical carcinogenesis has demonstrated that the carcinogenic potency of a compound is correlated with its mutagenic powers[1–4]; this suggests that DNA is the ultimate target of carcinogenic activation. Accordingly, we and others initiated a series of experiments designed to identify and study DNA segments in tumour cells whose alteration is critically important for oncogenic conversion[5–9].

An initial approach to this problem involved the transfer of tumour cell DNA into non-transformed NIH 3T3 mouse fibroblasts. Such experiments indicated that the phenotype of cellular transformation could be passed from cell to cell in this manner. Thus, the tumour DNA was able to induce foci of transformed cells in a recipient NIH 3T3 monolayer culture while DNA of normal, untransformed donor cells failed to do so[5–9]. These results demonstrated the existence of oncogenic factors which were present in tumour cell DNA and apparently absent from the DNA of normal cells.

That specific donor DNA sequences were involved in these phenomena was first indicated by studies examining the sensitivity or resistance of the biological activity of the DNA to treatment by various site-specific endonucleases[10,11], and was later directly demonstrated by molecular isolation of discrete transforming genes from human bladder carcinoma cell lines[12–14]. Use of clones of the bladder oncogene as sequence probes showed that the oncogene derived from a sequence of similar structure present in the normal human genome[12–14]. It appeared, therefore, that the bladder oncogene had arisen by the mutation of a normal cellular gene during the process of carcinogenesis. The present report addresses the nature of the alterations that activated the oncogene of the EJ human bladder carcinoma cell line.

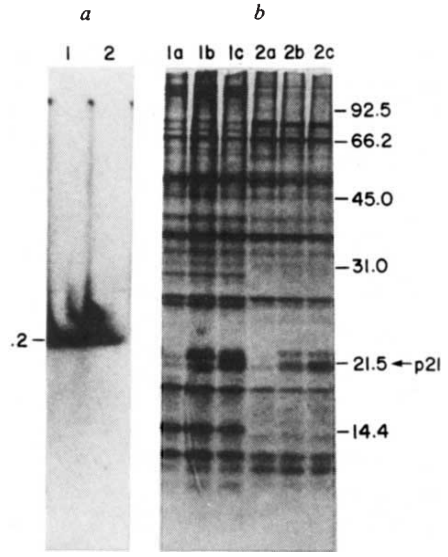

Fig. 1 Comparison of expression of the c-Ha-*ras* gene in normal human bladder epithelial cells with that in the EJ transformed bladder cell line. The normal bladder epithelial cells, strain HB1-5, were secondary and tertiary passage cultures derived from explants of a 5-month fetal human bladder. The cells were cultured as described[23]. *a*, The relative levels of c-Ha-*ras* specific RNA in the two cell types: lane 1, RNA from EJ cells; lane 2, RNA from Hbl-5 cells. Total polyadenylated RNA was prepared by the technique of Varmus *et al.*[40]; 4 μg of RNA was then fractionated by electrophoresis through formaldehyde-containing 2% agarose gels and transferred to nitrocellulose (B. Seed & D. Goldberg, manuscript in preparation). A *ras*-specific probe was prepared by cutting pEJ with *Bam*HI, fractionating the resultant fragments through a 1% agarose gel and extracting the 6.6-kb insert with NaI and glass beads. The nick-translated fragment (6.6×10^7 c.p.m. μg^{-1}) was annealed to the immobilized RNA[41,42]. Bands hybridizing to the probe were visualized by autoradiography. Molecular weights were determined by comparison with markers obtained from *in vitro* run-off transcription of the adenovirus late promoter[43]. *b*, A comparison of p21 proteins immunoprecipitated from cell lysates of EJ cell clones (lanes 1, a–c) and HB1-5 cells (lanes 2, a–c). Cultures were labelled with ^{35}S-methionine for 12 h. Lysates were then prepared and immunoprecipitated with non-immune serum (lanes 1a and 2a), a monoclonal antiserum (Y13-238) which precipitates the p21 encoded by Ha-MuSV but not the p21 encoded by Ki-MuSV (lanes 1b and 2b) or a monoclonal antiserum (Y13-259) which detects both the Ha-MuSV and the Ki-MuSV p21s (lanes 1c and 2c)[25,32]. 20×10^6 c.p.m. of lysate per sample were resolved by electrophoresis through a 12.5% SDS-polyacrylamide gel.

Comparison of this EJ bladder oncogene with the related cellular sequence (the proto-oncogene) was aided greatly by the subsequent discovery that this oncogene was homologous to the transforming gene of the rat-derived Harvey murine sarcoma virus[15–17]. This rat sarcoma virus gene, termed v-Ha-*ras*, had been acquired from the rat genome during the process of formation of the chimaeric viral genome[18,19]. Both the rat and human cellular homologues of v-Ha-*ras* have been isolated in the course of studies of this gene[20,21]. The human cellular homologue of the v-Ha-*ras* was found to correspond precisely to the normal antecedent of the EJ bladder oncogene[16].

From examination of the data in these cited reports, it was possible to make preliminary comparisons between the EJ oncogene[13] and its normal cellular counterpart (termed c-Ha-*ras*)[22]. For example, a molecular clone of the normal cellular gene did not induce foci when applied to NIH 3T3 monolayers, while a clone of the bladder oncogene exhibited a biological activity of $\sim5\times10^4$ focus-forming units per μg of transfected DNA[13,21]. This stark difference in function did not correlate with any apparent structural differences between the two clones. Rough restriction endonuclease site mapping of the EJ oncogene clone and the uncloned related human proto-oncogene indicated that the two were basically indistinguishable over the 6.6 kilobase (kb) of sequence which contained the transforming activity of the EJ oncogene[13]. Finer mapping was later made possible by the direct comparison of molecular clones of the two genes. We again found no differences using a series of different endonucleases, with the exception of a single difference 3′ (downstream) of the coding regions of the genes (data not shown). This latter difference was interpreted to represent a functionally silent polymorphism of the gene present in the human gene pool. Such polymorphisms of this gene have been documented independently by others[12].

These results presented an apparent paradox, since a drastic functional difference was exhibited by two structurally similar genes. We assumed that a minor alteration was responsible for the functional difference, and that this alteration could affect function in one of two ways: the alteration could involve a change in sequences regulating the expression of the gene; alternatively, the transformed phenotype could be due to changes in the protein-encoding portion of the gene. The first hypothesis would argue for up-regulation of transcription or translation of the gene, yielding high levels of an otherwise normal protein product, while the second model would suggest synthesis of an altered protein. Both types of alteration could also act in concert to create the observed difference in function.

Analysis of oncogene activity in normal and transformed bladder cells

If the significant difference between the oncogene and proto-oncogene were one of regulation, one would expect to see differences in levels of RNAs and proteins specified by these genes. To address this question, we first examined the EJ bladder carcinoma cells and their counterpart normal cells. Normal bladder epithelial cells were cultured from a specimen of normal human bladder as described[23], and were found to synthesize keratins, require a fibroblast feeder layer support, and to possess a limited lifespan in culture, as would be expected for normal epithelial cells (T. O'Connell and J. G. Rheinwald, personal communication). The cultures were free of human stromal fibroblasts, and were purified free of feeder cells before nucleic acid extraction. These cultured cells therefore provided a source of genetic material and RNA transcripts that were the normal counterparts of the bladder carcinoma genes and transcripts.

Total cellular RNA was prepared from both normal and transformed bladder cells. Transcripts were analysed by running the RNA on a formaldehyde gel, transferring it to a nitrocel-

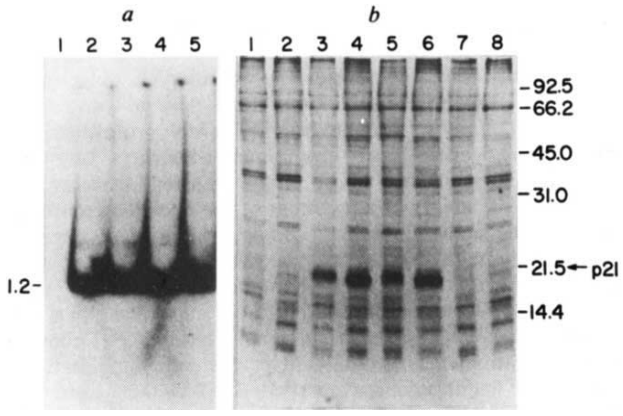

Fig. 2 Analysis of cells transfected with molecular clones of the EJ transforming gene (pEJ) or its normal cellular homologue (pEC). Transfections were carried out as previously described[44,45] using 75 μg NIH 3T3 carrier DNA, 500 ng pEJ or pEC DNA, and 50 ng pEcogpt DNA per 2×10^6 cells. Colonies resistant to mycophenolic acid were selected[26]. *a*, Analysis of total polyadenylated RNA from the four transfected cell lines. RNAs were isolated and analysed as described in Fig. 1. Lane 1, RNA from NIH 3T3 cells; lane 2, EJ/Gpt-2 cells; lane 3, EJ/Gpt-3 cells; lane 4, EC/Gpt-1 cells; lane 5, EC/Gpt-5 cells. *b*, A comparison of p21 proteins immunoprecipitated from cell lysates of the four lines transfected with pEJ or pEC. Cell lysates were prepared, immunoprecipitated and analysed as described in Fig. 1 legend. Immunoprecipitations were performed with non-immune serum (lanes 1, 2, 7, 8) or the monoclonal antiserum (Y13-238) which precipitates the Ha-MuSV p21 (lanes 3–6). Cell lysates were prepared from EJ/Gpt-2 (lanes 1, 3); EC/Gpt-1 (lanes 2, 4); EJ/Gpt-3 (lanes 5, 7) and EC/Gpt-5 (lanes 6, 8).

lulose filter, and probing the filter with nick-translated EJ oncogene clone. Figure 1*a* shows that similar levels of RNA were detected in the two cultures. The transcripts were of a size (1.2 kb) previously associated with the c-*ras* genes of other cell types[24].

The only known products of the *ras* genes are proteins of molecular weight ~21,000, referred to as p21. Monoclonal antisera against the v-Ha-*ras* p21 protein[25] were used to precipitate metabolically labelled protein lysates from both EJ and normal bladder cells. Control experiments ensured that the amounts of antibody used in this and subsequent experiments were in excess of that required to immunoprecipitate the antigen present. The data shown in Fig. 1*b* indicate that at least two bands of radiolabelled protein were specifically precipitated by the anti-p21 sera from normal bladder cells. Detailed examination of the protein pattern of the bladder carcinoma seen in this and other gels reveals a more complex array of bands. On close inspection we believe that four bands in total can be discerned. Data to be presented below clarify the origin of the novel p21 bands seen in the EJ tumour cells. After comparing the intensities of the p21 bands with those of non-specifically precipitated background bands, it became apparent that the total amount of p21 proteins of the normal and the tumour cells differed less than threefold.

It thus seems that increased levels of transcription are not responsible for the novel activity exhibited by the EJ oncogene. This conclusion rests in part on the fact that in the hybridization conditions used here, the oncogene probe reacts exclusively with transcripts of the human c-Ha-*ras*-1 gene. Interpretation of the protein data is less clear. It is apparent that both cells have comparable levels of proteins that are reactive with the Harvey-specific serum, and that these proteins can be collectively termed 'p21'. The nature of these proteins can only be addressed by other studies, presented in part below.

Analysis of gene activity in cells transfected with cloned DNAs

It remained possible that the bladder epithelial cells were not, as we believed, representatives of the normal precursors of the bladder carcinoma cells. Such a possibility might cloud interpretation, as a *ras* gene could be expressed at a high level in one cell type without inducing transformation, and only achieve this phenotype when inappropriately expressed in a second cell type. We therefore wished to measure the levels of transcription and translation of the two genes in the same cellular background.

We therefore introduced molecular clones of both genes into NIH 3T3 cells. Colonies acquiring the EJ oncogene could be readily identified by their transformed morphology. However, cells acquiring clones of the normal allele were not identifiable by any obvious change in behaviour. Because of this, we co-transfected a clone of the dominant selectable *Ecogpt* gene[26] into NIH 3T3 cells together with a 10-fold excess of either the cloned EJ oncogene (pEJ) or the cloned proto-oncogene (pEC). In each case, colonies were selected for resistance to mycophenolic acid imparted by acquired *Ecogpt* genes[26]. This strategy was used since the introduction of a non-selected DNA segment can be ensured by co-transfection with a selectable gene[27]. In the present case, 75% of the mycophenolic acid-resistant colonies deriving from co-transfection of *Ecogpt* and pEJ were seen to be morphologically transformed; as expected, none of the colonies emerging after co-transfection with pEC was transformed.

The cellular DNA of both classes of colonies was analysed for the presence of pEC or pEJ sequences. DNA was cleaved with the restriction enzyme *Bam*HI, which would be expected to liberate a 6.6-kb fragment from each intact copy of the cloned oncogene or proto-oncogene. The normal mouse homologue of the *ras* gene hybridizes only weakly to the pEJ probe[16], and so its presence does not obscure the analysis. To ensure that transfected pBR322 sequences would not interfere with interpretation of the data, *ras*-specific sequences were prepared from pEJ and used as probe; 75% of non-transformed colonies transfected with the proto-oncogene and all of the transformed, oncogene-transfected colonies showed the presence of pEJ-homologous sequences migrating at 6.6 kb (data not shown).

We selected for further analysis two cell lines containing intact copies of the oncogene and two lines containing an approximately equal number of intact copies of the proto-oncogene. Photographs of these cell lines are shown in Fig. 3; 4×10^6 cells of each of these lines were injected into 6-week-old NSF mice. Each of the pEJ transfected lines elicited tumours in 5/5 mice, while none of the mice injected in parallel with pEC transfectants developed tumours.

Total cellular RNA was prepared from all four transfected cell lines. The RNA preparations were then run on a formaldehyde gel, transferred to nitrocellulose filters, and probed with the *ras*-specific DNA. As shown in Fig. 2*a*, the level of *ras*-specific RNA in cells containing the oncogene was comparable with those carrying the transfected proto-oncogene. We also examined the levels of p21 in these cell lines. Figure 2*b* shows that monoclonal antiserum against p21 precipitates similar amounts of p21 protein in pEC and pEJ transfected cells.

These data do not address the question of whether the two cloned genes are transcribed at the same rates in these cells, as it remains formally possible that few of the acquired copies of the pEJ are active in these particular pEJ-transfected cells, while all copies of the transfected pEC gene in the other cells might be active. In such a case, the comparable levels of protein and RNA observed would not accurately reflect the instrinsic transcriptional activities of the two genes.

However, one point emerges with clarity from these experiments: a certain level of EJ-specified p21 induces transformation, while a comparable if not higher level of the proto-oncogene-specified p21 has no effect on cellular phenotype. Since the p21 proteins are the only apparent gene products encoded by these genes, we conclude that the difference in function between the EJ oncogene and the proto-oncogene must derive from structural alterations in the p21 protein. Conversely, regulatory alterations do not seem critical to the transforming activity of the oncogene.

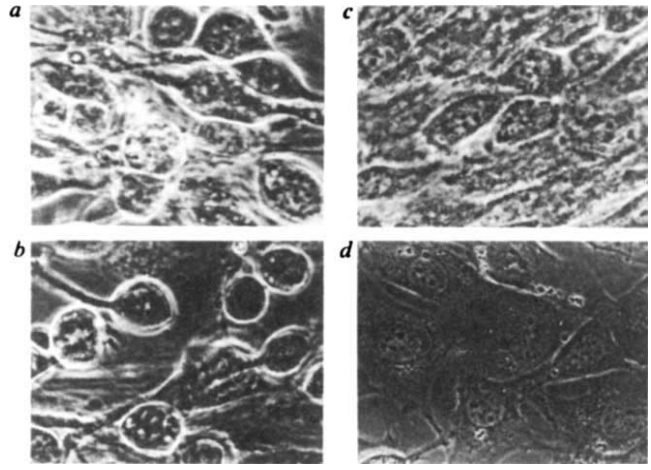

Fig. 3 Photographs of cell lines transfected with pEJ or pEC through a phase contrast microscope (×300). Cell lines were derived as described in Fig. 2 legend. Cell lines transfected with pEJ are shown at confluence (EJ/Gpt-2, *a*) and subconfluence (EJ/Gpt-3, *b*). Cell lines transfected with pEC are also shown at confluence (EC/Gpt-5, *c*) and subconfluence (EC/Gpt-1, *d*).

Detection of differences in the size of the oncogene product

In view of the above conclusion, new importance was attached to the previously detected slight variations in migration rates of the p21 proteins from different cells (Figs 1*b* and 2*b*). We therefore re-analysed the p21 proteins after shorter periods of metabolic labelling, which we expected would highlight these differences more clearly. Data shown in Fig. 4*a* indicate that the pEJ and pEC transfectants each exhibit two bands of p21. Kinetic labelling experiments indicate that in each case the more slowly migrating band behaves as a kinetic precursor to the more rapidly migrating band (manuscript in preparation). Comparable data on the p21 protein of v-Ha-*ras* previously indicated that the higher band underwent post-translational cleavage to yield its lower, more rapidly migrating partner[28].

Further examination of Fig. 4*a* reveals that the higher molecular weight p21 protein of the pEJ transfectant migrates slower than the higher molecular weight protein of the pEC transfectant and also that the lower molecular weight p21 of the pEJ transfectant migrates slower than the lower molecular weight p21 of the pEC transfectant. The relative migration rates of the these proteins, and the relationships we impute to them from kinetic data and previously published experiments[28] are indicated schematically in Fig. 4*a*.

As none of the p21 proteins seems to be phosphorylated to any extent (data not shown), the differences in migration between the pEC and pEJ proteins are most readily attributed to alterations in the number of amino acids or to changes in conformation. The data and the schematization of Fig. 4*a* may provide an explanation for the complexity of p21 proteins seen in normal and transformed bladder cells (Fig. 1*b*): the normal cells exhibit two bands, reflective of the expression of a proto-oncogene; the carcinoma cells appear to exhibit four bands, two being specified by the oncogene of these cells, and two by the normal, proto-oncogene of the other, homologous chromosome.

Genetic mapping of the functionally altered region of the oncogene

To determine whether the observed physical difference between p21 proteins reflected a functionally important change involved in the process of transformation, we set out to supplement these results with an independent series of experiments designed to localize genetically the regions of the oncogene that specify the altered migration rate of the protein and the change in gene function. These experiments depended on *in vitro* homologous recombination between clones of the two genes. The experimental design was to excise a restriction fragment out of the oncogene and use it to replace the homologous piece of the proto-oncogene. At the same time, the reciprocal construction was carried out by splicing the fragment of the proto-oncogene into the oncogene. These recombinant constructs were then tested for transforming ability in the transfection assay. We looked for the ability of a fragment of the oncogene to impart transforming activity when placed in the midst of a proto-oncogene clone, and conversely, in the reciprocal recombination, for the loss of activity when the corresponding proto-oncogene fragment was inserted into the oncogene clone. The constructions are shown schematically in Fig. 5. Following *in vitro* ligation, the recombinant clones were amplified and mapped to confirm their construction. Only then were they transfected to NIH 3T3 monolayers.

It was vital that we should be able to verify that the transforming clones were indeed chimaeras of mixed pEJ and pEC origin, rather than contaminants of one origin or the other. This was

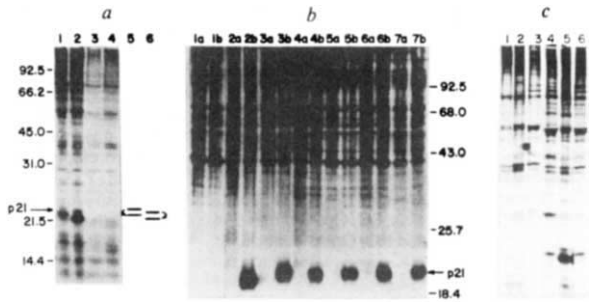

Fig. 4 Comparison of the mobility of p21 proteins encoded by the EJ bladder carcinoma transforming gene and its normal homologue. *a*, Immunoprecipitations from cells transfected with pEJ or pEC. Cells were metabolically labelled with ^{35}S-methionine for 3 h; cell lysates were prepared, immunoprecipitated and analysed as described in Fig. 1 legend. Cell lines were derived as described in Fig. 2 legend. Lysates from the cell line EJ/Gpt-3 (lanes 1, 3) and from the cell line EC/Gpt-1 (lanes 2, 4) were precipitated with the monoclonal anti-p21 antiserum Y13-238 (lanes 1, 2) or with non-immune serum (lanes 3, 4). Schematic diagrams (lane 5, EJ/Gpt-3; lane 6, EC/Gpt-1) show both the relative positions of the detected p21 bands and the relationships of those bands (arrows) based on kinetic data (not shown) and previously published experiments[28]. *b*, Analysis of the migration of p21 proteins immunoprecipitated from cells transfected with *in vitro* recombinants of the EJ oncogene (pEJ) and its homologous proto-oncogene (pEC). NIH 3T3 cells transformed with the EJ bladder tumour oncogene, its normal proto-oncogene, or recombinants between the two genes were first biologically cloned in 0.35% agar and then metabolically labelled with ^{35}S-methionine for 18 h. Lysates were prepared and immunoprecipitated (5×10^6 c.p.m. of TCA-precipitable counts) by a monoclonal antibody that detects the p21 encoded by Ha-MuSV but not the p21 encoded by Ki-MuSV (Y13-172)[25,32]. Dissolved immunoprecipitates were then resolved by electrophoresis in a 12% SDS-polyacrylamide gel. Lanes 1a–7a, no antibody; lanes 1b–7b, anti-Harvey p21 monoclonal antibody. Cell lysates were from: NIH 3T3 cells (lane 1); cells transformed with the proto-oncogene (the 3-kb *Sac*I fragment of the pEC proto-oncogene that has been activated by fusion with a retrovirus LTR transcriptional promoter described in ref. 35) (lane 2); cells transformed with the EJ oncogene (6.6-kb fragment in pBR) (lane 3); clone 511-74, transformed with the ligation of proto-oncogene 1-kb *Sac*I fragment to EJ oncogene 3-kb *Sac*I fragment (construction b2, Fig. 5) (lane 4); cells transformed with ligations of a fragment of the EJ oncogene extending from the *Xho*I site to the second *Bst*EII site to a clone of the proto-oncogene from which the homologous fragment had been removed (construction e2, Fig. 5) (lanes 5 and 6); and cells transformed with the ligation of the EJ oncogene to the left of the *Kpn*I site to the proto-oncogene to the right of this site (construction a2, Fig. 5) (lane 7). *c*, Analysis of the migration of the p21 proteins immunoprecipitated from cells transfected with the *in vitro* recombinant in which the 350-bp *Xma*I–*Kpn*I fragment of the pEJ oncogene clone was inserted into the corresponding region of the pEC proto-oncogene clone. Cells were metabolically labelled with ^{35}S-methionine for 3 h; cell lysates were prepared, immunoprecipitated and analysed as described in Fig. 1 legend. Cell lines EC/Gpt-1 and EJ/Gpt-3 were derived as described in Fig. 2 legend. The *in vitro* recombinant was constructed as described in Fig. 5 legend, part h2. The resulting construct was transfected as described in Fig. 2 legend and a transformed focus was picked and expanded. Immunoprecipitations were performed with non-immune serum (lanes 1–3) or with the monoclonal antiserum (Y13-238) which precipitates the Ha-MuSV p21 (lanes 4–6). Cell lysates were prepared from EJ/Gpt-3 (lanes 1 and 4); EC/Gpt-1 (lanes 2 and 5); and the *Xma*I–*Kpn*I recombinant transfected cell line (lanes 3 and 6).

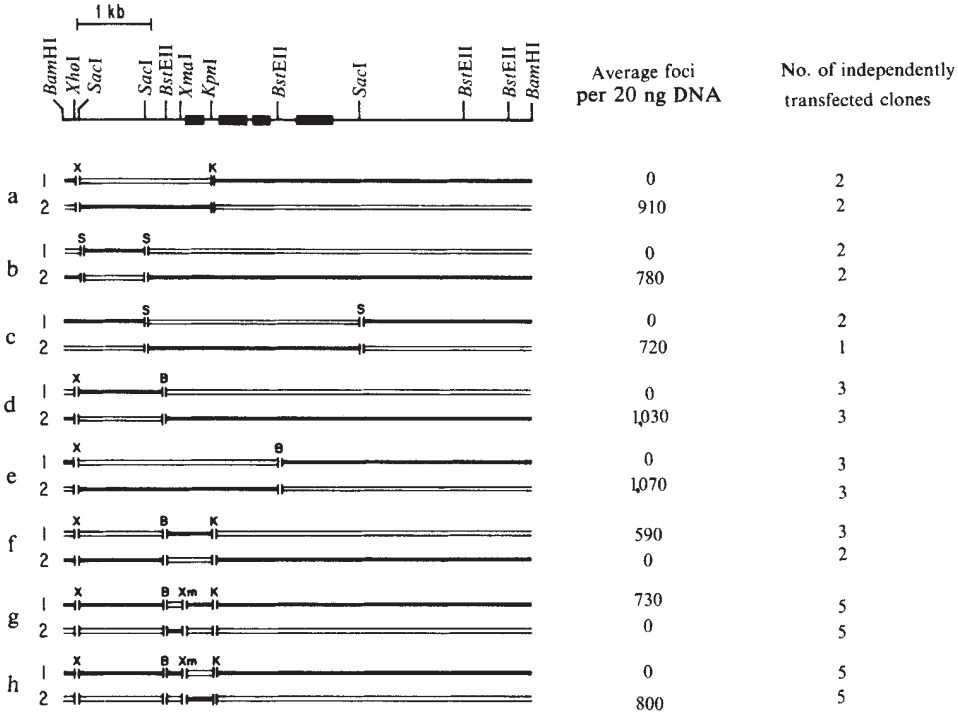

Fig. 5 Transfection data and structures of *in vitro* genetic recombinants between the molecular clones of the EJ transforming gene (pEJ) and its normal cellular homologue (pEC). The restriction map shows the cleavage sites for various enzymes within the 6.6-kb *Bam*HI insert in pBR322. All sites specific for the enzymes are shown except for *Xma*I which cuts in several other places which have not been well characterized. The site shown is the only *Xma*I site between the first *Bst*EII site and the *Kpn*I site. The solid boxes on the map show the locations of coding exons. pEJ/pEC chimaeras are shown, with segments derived from pEJ shown as solid bars and segments from pEC shown as open bars. pEJ and pEC were cleaved with the indicated enzymes either to completion or in a partial digest as required to obtain each indicated fragment. The products were separated by electrophoresis through 1.2% agarose and eluted by melting in NaI and adsorbing to glass beads. The fragment containing pBR322 was then treated with calf intestinal phosphatase. The indicated fragments were joined either with the enzyme T4 DNA ligase or in a mock ligation without enzyme. Constructs a–e were made in bimolecular ligations. Constructs in f were made by mixing the three fragments simultaneously and in g and h by mixing the four fragments simultaneously. The ligation mixtures were directly transformed into the HB101 strain of *Escherichia coli*. Only when colonies from mock ligations were less than 2% of the ligations were colonies analysed for the presence of clones with appropriate restriction maps; 20 ng of each clone were transfected to NIH 3T3 cells as described in Fig. 2 legend and then carried without selection until foci were visualized in 10–14 days. Results of the transfections are shown in the first column. The second column shows the number of independent bacterial colonies screened and then transfected into NIH 3T3 cells.

done in three ways. In the simpler constructions, involving ligations of two fragments at a time, we verified results obtained with amplified recombinant clones by directly transfecting the unamplified products of ligation reactions and of mock ligations containing isolated fragments not treated with the ligase. A second confirmation depended on the fact that the plasmids pEJ and pEC contained their respective cellular genes inserted in the pBR322 plasmid vector in opposite orientations. Thus, we could learn the origin of one parent of a recombinant by diagnostic restriction digests of the flanking plasmid regions. Since contaminating pEC could itself not give a false positive result (as it is not transforming), any active clone carrying proto-oncogene flanking sequences must have arisen as a consequence of acquisition of portions of the transforming gene. Finally, we confirmed all of our results with several independent clones obtained from a ligation reaction. The products of a ligation were used only if mock ligation of the same fragments gave no more than 2% of the bacterial colonies seen on use of the DNA ligase.

The results of transfecting the recombinant clones are summarized in Fig. 5. As can be seen, we ultimately identified a genetic region 350 nucleotides long which, when transferred from the oncogene to a corresponding region in the proto-oncogene, is able to impart activity to the latter. This region extends from the first *Xma*I endonuclease site to the *Kpn*I site (Fig. 5); 55% of this region consists of the first coding exon, 10% is 5′ to the coding region and 35% is part of the first intron.

Detection of differences in size of the gene product from the *in vitro* recombinants

In experiments described above, we had identified a difference in the migration of the p21 protein encoded by the oncogene and the proto-oncogene. Having now determined a short region of the gene which contained the transforming lesion, it became important to ascertain whether the region also contains the specificity for the altered protein size. We therefore carried out immunoprecipitations on cells transfected with the products of the *in vitro* recombinants. As seen in Fig. 4*b*, the protein brought down in lysates from *Xho*I–second *Bst*II site, *Sac*I–*Sac*I, and *Xho*I–*Kpn*I recombination transfectants all co-migrate with the EJ protein and have a mobility which differs from that of the EC protein. Similarly, Fig. 4*c* demonstrates this result for the *Xma*I–*Kpn*I recombinants. The lysates analysed in panel 4*b* were from cultures that were labelled for 18 h, resulting in high levels of label in the lower molecular weight forms of the p21, and undetectable amounts of label in the kinetically unstable higher molecular weight forms. We conclude that the phenotypes of oncogenic transformation and altered electrophoretic migration co-segregate, and the two are encoded by the same 350-bp segment of DNA. As indicated below, the altered migration rate is probably a reflection of a functionally important alteration of the protein.

Nucleotide sequence of the transforming lesion

Having identified a short fragment of biological significance, we then sequenced this region of the oncogene and the proto-oncogene. Figure 6 compares the sequences of the two genes in this segment obtained by sequencing both strands. The DNA sequence described in the figure was obtained by the forward and backward dideoxy DNA sequencing technique of Seif *et al.*[29] and by the chemical procedure of Maxam and Gilbert[30].

The difference between the two segments is in the p21 encoding region of the first known exon, specificially 60 nucleotides from the *Xma*I cleavage site. It occurs in a triplet that encodes glycine in the normal rat (M. Ruta *et al.*, unpublished data) and human c-Ha-*ras* genes. The sequence observed in the EJ oncogene causes conversion to a valine. As we discuss below, we believe that this alteration is responsible for the alteration in function of the p21 protein, and for the oncogenic activation of the c-Ha-*ras* gene that occurred in the EJ bladder carcinoma.

Alteration of a diagnostic restriction endonuclease cleavage site

A second independent consequence of the single base change was the alteration in the cleavage sites of two different site-specific endonucleases. The sequence GCCGGC occurs in the

proto-oncogene, and thus represents the recognition site of the endonuclease *Nae*I. It also contains the CCGG recognition site of the endonuclease *Hpa*I. Both of these are changed in the oncogene, the equivalent sequence of which reads GCCGTC. The *Nae*I endonuclease is the more useful of the two since it cleaves DNA less frequently, and we used it to independently verify the two sequences. As expected, the pEC clone exhibited one more cleavage site in its inserts than its pEJ counterpart (data not shown). Perhaps more useful was the retrospective verification of the *in vitro* recombinant clones. The allele specifying transformation and abnormal p21 migration was seen to precisely co-segregate with the allele disallowing *Nae*I cleavage at this site (data not shown).

Discussion

Use of gene transfer and molecular cloning had previously made it possible to localize an important lesion of the EJ bladder carcinoma genome to a 6.6-kb DNA segment constituting only 10^{-6} of the genetic content of the tumour cell. The segment appears to contain the entire transforming activity previously associated with the whole tumour cell DNA. This oncogene is an important, but perhaps not the sole, determinant of the transformed phenotype of these carcinoma cells. Sequence hybridization showed that this oncogene was related in structure to a similar sequence existing in the normal cellular genome[12–15]. It appeared that the oncogene arose from the activation of these normal sequences, and that this activation occurred during the process of carcinogenesis which led to formation of the original EJ carcinoma. Experiments described in this report were designed to resolve and define the critical differences that distinguish the oncogene from its proto-oncogene precursor.

It was apparent that a definition of these differences could not be achieved by simple comparison of the sequences of the two genes. As the oncogene and its normal proto-oncogene counterpart sequence were derived from the DNAs of separate individuals, most sequence differences might reflect naturally occurring, silent polymorphisms at this locus. Other sequence differences could be consequences of mutational insults suffered during carcinogenesis, which were silent functionally and thus of no importance to the activation process. These considerations caused us to undertake the *in vitro* recombination experiments in order to narrow the area of functional importance to a segment so small that it would probably contain few if any silent mutations. Only after narrowing the functional segment to 350 bp did we initiate DNA sequence studies.

The sequence difference that we observed in this region could have only one consequence for the structure of the p21 protein—a simple amino acid substitution leading to the replacement of a glycine by a valine residue. This substitution appears to represent the critical agent of the conversion of the proto-oncogene into an active oncogene.

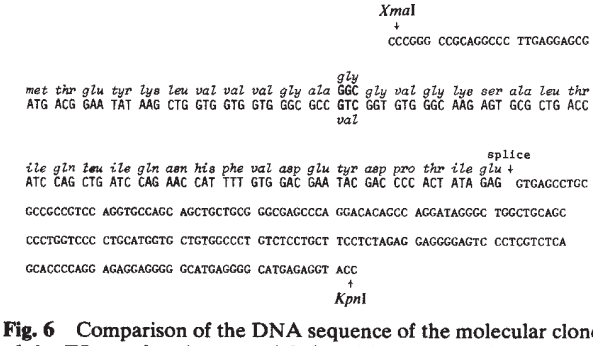

Fig. 6 Comparison of the DNA sequence of the molecular clone of the EJ transforming gene (pEJ) and its non-transforming cellular homologue (pEC). Sequence data between the restriction enzyme sites *Xma*I and *Kpn*I are shown (see Fig. 5). The coding DNA strand is printed as is the inferred amino acid sequence. For coding regions where pEJ and pEC differ, both sequences are indicated. Sequences were determined by the forward and backward dideoxy DNA sequencing technique of Seif *et al.*[29] and by the chemical procedure of Maxam and Gilbert[30].

Such a simple substitution might not be expected to effect a profound change in the function of the p21 protein. However, several considerations seem to confer importance on the structural alteration. The first stems from comparison of this domain of p21 encoded by the human c-Ha-*ras*-1 gene, the rat c-Ha-*ras*-1 gene (Ruta *et al.*, unpublished results) and the v-Ha-*ras* oncogene of Harvey sarcoma virus[31]. The 37-residue long amino acid sequences encoded by the first exons of the two cellular genes are identical, indicating strict evolutionary conservation of this region. However, analysis of the Harvey sarcoma virus oncogene reveals that it deviates from its direct rat cellular antecedent in only one position in this domain, a glycine to arginine conversion at precisely the same residue that is altered in the EJ oncogene. Thus, we speculate that alteration of this critical residue was important both in the activation of the v-Ha-*ras* gene from its rat cellular precursor, and in the activation of the EJ bladder oncogene from its normal human counterpart. Moreover, a similar alteration may have been significant in the oncogenic activation of another member of the *ras* gene family, the v-Ki-*ras* gene of Kirsten murine sarcoma virus. The Kirsten transforming gene is closely related to the v-Ha-*ras* gene[32]. The only difference between the p21 of v-Ki-*ras* and that of v-Ha-*ras* in the first 36 amino acids is at position 12, where the residue in Kirsten is serine[33], again at precisely the same site as altered in the EJ and Harvey sarcoma virus encoded p21s. While sequence information is not available on the cellular homologue of v-Ki-*ras*, we speculate that a conversion from a glycine to a new amino acid residue at position 12 may also have been involved in the activation of this *ras* oncogene.

A second consideration stems from examination of the specific amino acid changes observed in these cases. In both instances, glycine is replaced by an amino acid having a relatively bulky side chain. Glycine is an anomaly among the 20 amino acids, because it lacks a side chain. Thus, it is able to participate in extremes of bending and folding of the polypeptide backbone and is the strongest breaker of α-helices[34]. Thus, replacements of glycine by valine or arginine represent abrupt changes in the local stereochemistry of a protein.

We speculate that the loss of glycine at residue 12 causes a significant change in an essential domain of the p21 protein. One consequence of this change may be a conformational shift of the protein, leading in turn to the aberrant electrophoretic migration or processing of the p21 proteins. A second, more important consequence is a profound effect on the function of the p21 protein. This alteration probably affects interaction of the p21 with cellular targets. A precedent exists for other single amino acid changes having profound effects on cellular and organismic physiology. The most well known of these is the sickle-cell syndrome, in which a glutamine to valine conversion affects the solubility of haemoglobin within erythrocytes.

The importance we attach to alteration of protein structure would seem to contradict a series of experiments of recent years that indicate up-regulation as the pivotal event in carcinogenesis. These experiments include the activation of the *myc* proto-oncogene occurring during leukaemogenesis of avian retroviruses[35,36]; and the demonstration that *in vitro* fusion of a retroviral long terminal repeat (LTR) promoter and a cellular proto-oncogene results in an actively transforming gene[20,37,38]. These latter experiments are particularly germane, since some of them demonstrate activation of clones of the rat and human c-Ha-*ras* proto-oncogenes[20,38]. It is unlikely that the protein-encoding sequences of these c-Ha-*ras* genes have undergone any structural changes during construction of these viral–cellular chimaeras. Rather, it appears that the only essential difference between the proto-oncogenes and their LTR-activated counterparts lies in rates of expression. This means that the *ras* proto-oncogene can be activated by a second independent mechanism, in principle as effective in creating an oncogene as the one described here.

We point out that oncogenes of other tumours have also been traced to *ras* genes. Specifically, colon and lung carcinomas

(ref. 15 and M. McCoy, J. J. Toole, E.H.C. and R.A.W., in preparation) as well as fibroblastic tumours (L. Parada and R.A.W., unpublished results) have been found to carry oncogenes derived from activation of cellular Ki-*ras* genes. As discussed above, the N-terminal regions of the Ha-*ras* and Ki-*ras* proteins are extremely similar, and the Ki-*ras* proteins of many of these tumours also exhibit aberrant electrophoretic migration rates (manuscript in preparation). We predict that activation of many of these other oncogenes will depend on structural alterations very similar to those reported here.

Most amino acid sequence alterations are either neutral or deleterious to protein function. Few are able to actively potentiate the normal functions of a protein. We suggest that only a small number of sites on the p21 protein can be altered in a fashion leading to oncogenic activation. Most mutations will affect other residues whose alteration will be unproductive for oncogenic conversion. The target for oncogenic conversion may therefore be exceedingly small, and may even be confined to the Gly-12 codon. In such a case, the restriction site for *Nae*I, which totally spans that codon, may provide an ideal diagnostic tool for detecting a critical change in the genome.

The present data suggest that the alteration of one nucleotide in one bladder cell leads to the creation of an activated oncogene. There are three possible point mutations at this position, and it is perhaps not coincidental that the G-T transversion observed here is precisely that mutation favoured by many suspected bladder carcinogens[39]. The oncogene resulting from this mutation was probably an important determinant in the subsequent outgrowth of a lethal neoplasm. The point mutation implicated as a central event in this oncogenic transformation represents the first demonstration of a lesion in cellular DNA whose occurrence is directly related to the carcinogenic process.

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1. McCann, J., Choi, E., Yamasaki, E. & Ames, B. N. *Proc. natn. Acad. Sci. U.S.A.* **72,** 5135–5139 (1975).
2. McCann, J. & Ames, B. N. *Proc. natn. Acad. Sci. U.S.A.* **73,** 950–954 (1976).
3. Bridges, B. A. *Nature* **261,** 195–200 (1976).
4. Bouck, N. & diMayorca, G. *Nature* **264,** 722–727 (1976).
5. Shih, C., Shilo, B., Goldfarb, M. P., Dannenberg, A. & Weinberg, R. A. *Proc. natn. Acad. Sci. U.S.A.* **76,** 5714–5718 (1979).
6. Cooper, G. M., Okenquist, S. & Silverman, L. *Nature* **284,** 418–421 (1980).
7. Shih, C., Padhy, L. C., Murray, M. J. & Weinberg, R. A. *Nature* **290,** 261–264 (1981).
8. Krontiris, T. G. & Copper, G. M. *Proc. natn. Acad. Sci. U.S.A.* **78,** 1181–1184 (1981).
9. Perucho, M. *et al. Cell* **27,** 467–476 (1981).
10. Lane, M. A., Sainten, A. & Cooper, G. M. *Proc. natn. Acad. Sci. U.S.A.* **78,** 5185–5189 (1981).
11. Shilo, B. & Weinberg, R. A. *Nature* **289,** 607–609 (1981).
12. Goldfarb, M., Shimizu, K., Perucho, M. & Wigler, M. *Nature* **296,** 404–409 (1982).
13. Shih, C. & Weinberg, R. A. *Cell* **29,** 161–169 (1982).
14. Pulciani, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **79,** 2845–2849 (1982).
15. Der, C., Krontiris, T. G. & Cooper, G. M. *Proc. natn. Acad. Sci. U.S.A.* **79,** 3637–3640 (1982).
16. Parada, L. F., Tabin, C. J., Shih, C. & Weinberg, R. A. *Nature* **297,** 474–479 (1982).
17. Santos, E. *et al. Nature* **298,** 343–347 (1982).
18. Scolnick, E. M. & Parks, W. P. *J. Virol.* **13,** 1211–1219 (1974).
19. Shih, T. Y. *et al. J. Virol.* **27,** 45–55 (1978).
20. DeFeo, D. *et al. Proc. natn. Acad. Sci. U.S.A.* **78,** 3328–3332 (1981).
21. Chang, E. H., Gonda, M. A., Ellis, R. W., Scolnick, E. M. & Lowy, D. R. *Proc. natn. Acad. Sci. U.S.A.* 4848–4852 (1982).
22. Ellis, R. W. *et al. J. Virol.* **36,** 408–420 (1980).
23. Wu, Y-J., *et al. Cell* (submitted).
24. Ellis, R. W., DeFeo, D., Furth, M. E. & Scolnick, E. M. *Cell* (in the press).
25. Furth, M. E., Davis, L. J., Fleurdelys, B. & Scolnick, E. M. *J. Virol.* **43,** 294–304 (1982).
26. Mulligan, R. & Berg, P. *Proc. natn. Acad. Sci. U.S.A.* **78,** 2072–2076 (1981).
27. Wigler, M. *et al. Cell* **16,** 777–785 (1979).
28. Shih, T. Y. *et al. J. Virol.* **42,** 253–261 (1982).
29. Seif, I., Khoury, G. & Dhar, R. *Nucleic Acids Res.* **8,** 2225–2238 (1980).
30. Maxam, A. H. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74,** 560–564 (1977).
31. Dhar, R. *et al. Science* **217,** 934–936 (1982).
32. Shih, T. Y., Weeks, M. O., Young, H. A. & Scolnick, E. M. *Virology* **96,** 64–79 (1979).
33. Tsuchida, N., Ryder, T. & Ohtsubo, E. *Science* **217,** 937–939 (1982).
34. Cantor, C. R. & Schimmel, P. R. in *Biophysical Chemistry* Vol. 1, 303–304 (Freeman, San Francisco, 1980).
35. Neel, B. G., Hayward, W. S., Robinson, H. L., Fang, J. & Astrin, S. M. *Cell* **23,** 323–334 (1981).
36. Payne, G. S. *et al. Cell* **23,** 311–322 (1981).
37. Blair, D. G. *et al. Science* **212,** 941–943 (1981).
38. Chang, E. J., Furth, M. E., Scolnick, E. M. & Lowy, D. R. *Nature* **297,** 479–483 (1982).
39. Swenson, D. H. & Kadlubar, F. F. in *Microbial Testers Probing for Carcinogenesis* (ed. Felkner, I. C.) 3–33 (Dekker, New York, 1981).
40. Varmus, H. E., Quintrell, N. & Ortiz, S. *Cell* **25,** 23–36 (1981).
41. Rigby, P. W. *et al. J. molec. Biol.* **13,** 237–251 (1977).
42. Wahl, G. M., Stern, M. & Stark, G. R. *Proc. natn. Acad. Sci. U.S.A.* **76,** 3683–3687 (1979).
43. Manley, J. L. *et al. Proc. natn. Acad. Sci. U.S.A.* **77,** 3855–3859 (1980).
44. Graham, F. L. & van der Eb, A. J. *Virology* **52,** 456–471 (1973).
45. Andersson, P., Goldfarb, M. P. & Weinberg, R. A. *Cell* **16,** 63–75 (1979).

A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene

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The genetic change that leads to the activation of the oncogene in T24 human bladder carcinoma cells is shown to be a single point mutation of guanosine into thymidine. This substitution results in the incorporation of valine instead of glycine as the twelfth amino acid residue of the T24 oncogene-encoded p21 protein. Thus, a single amino acid substitution appears to be sufficient to confer transforming properties on the gene product of the T24 human bladder carcinoma oncogene.

DNA-MEDIATED gene transfer techniques have made it possible to identify the presence of dominant transforming genes (oncogenes) in a variety of human tumours (for review see ref. 1). Although only a small number of human tumour DNAs have been shown to be capable of transforming normal cells in transfection assays, oncogenes have been detected in tumours representative of each of the major forms of human cancer[2–8]. To date, more than 10 different human oncogenes have been identified. One of them, present in T24 and EJ bladder carcinoma cell lines, has been isolated by molecular cloning techniques[8–10]. Preliminary characterization of this oncogene has revealed that it is small (less than 4.6 kilobase, kb), and that it has not undergone major genetic rearrangements[9–11]. Comparative analysis of this bladder carcinoma oncogene with retroviral transforming (*onc*) genes has revealed that an internal fragment of the T24 oncogene is closely related to the *onc* genes of