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Spontaneous activation of a human proto-oncogene

(malignant transformation/c-*has/bas* gene/point mutation/12th codon/aspartic acid)

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ABSTRACT **It has been recently shown that malignant activation of the c-*has/bas* proto-oncogene in T24 human bladder carcinoma cells was mediated by a single point mutation. A deoxyguanosine located at position 35 of the first exon of this proto-oncogene was substituted by thymidine. These findings predicted that the resulting oncogene would code for a structurally altered p21 protein containing valine instead of glycine as its 12th amino acid residue. We now report the spontaneous activation of the human c-*has/bas* proto-oncogene during transfection of NIH/3T3 cells. As in T24 cells, this *in vitro* activated oncogene also acquired malignant properties by a single point mutation. In this case we have detected a G→A transition, which occurred at the *same* position as the mutation responsible for the activation of the T24 oncogene. These results predict that the p21 protein coded for by the spontaneously activated c-*has/bas* gene will incorporate aspartic acid as its 12th amino acid residue. Computer analysis of the secondary structure of c-*has/bas*-encoded p21 proteins indicates that substitution of the glycine residue located at position 12, not only by aspartic acid or valine but also by any other amino acid, would result in the same structural alteration. These findings indicate that a specific conformational change is sufficient to confer transforming properties to this p21 protein. Moreover, they predict that any mutation affecting the coding properties of the 12th codon of the c-*has/bas* proto-oncogene will lead to its malignant activation.**

Vertebrate cells contain a family of evolutionarily conserved proto-oncogenes (generically designated *ras*), some of which have been shown to acquire malignant properties upon recombination with retroviral sequences (1) and may be involved in the development of certain human neoplasias (for review, see ref. 2). For instance, an activated form of the c-*has/bas* proto-oncogene, whose sequences were transduced into the *onc* genes of the Harvey and BALB strains of murine sarcoma viruses (3), has been identified in the T24 human bladder carcinoma cell line (4–6). Comparative analysis of molecular clones containing the T24 oncogene (7–9) and its corresponding proto-oncogene (6, 10) present in normal human cells has led to the surprising discovery that a single point mutation is responsible for its malignant properties (11–14). A G→T transversion located at position 35 of the T24 oncogene coding sequences results in the incorporation of valine instead of glycine as the 12th amino acid residue of the p21 protein, the gene product of the T24 human oncogene. Thus, a conformational alteration in this protein appears to be responsible for its transforming properties (11–14).

We now report the spontaneous activation of the human c-*has/bas* proto-oncogene. This event occurred during the course of transfection assays without participation of known carcinogenic agents. Acquisition of transforming properties by this proto-oncogene was mediated by a single point mutation, which occurred at the *same* nucleotide (a deoxyguanosine residue at position 35 of the coding sequences of the c-*has/bas* proto-oncogene), whose substitution led to the generation of the T24 bladder carcinoma oncogene.

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MATERIALS AND METHODS

The experimental procedures have been described in detail elsewhere (6, 12). They include transfection assays (15, 16), Southern blot analysis of DNA (17), molecular cloning (18), and nucleotide sequence analysis (19). When necessary, specific experimental details are given in the text and in the figure legends.

RESULTS

***In Vitro* Activation of the Human c-*has/bas* Proto-Oncogene.** We have recently found that the human c-*has/bas* proto-oncogene contains all of the necessary information to drive NIH/3T3 cells to malignancy. However, this biological effect is only observed when multiple (>30) copies of the human proto-oncogene become integrated in the NIH/3T3 cellular genome (unpublished data). Because the malignant phenotype of these transformants could not be transmitted in additional cycles of transfection, it appears that it is conferred by the combined effect of the multiple copies of the normal c-*has/bas* gene rather than by one or a few copies of an activated form of this proto-oncogene (unpublished data).

However, during the course of these experiments we observed that DNA isolated from one of these proto-oncogene derived-transformants (designated 115-14) induced the appearance of a single focus of morphologically transformed cells when transfected into NIH/3T3 cultures. The 115-14 cell line is an NIH/3T3 clone transformed by multiple (50–60) copies of λbc-N DNA, a Charon 4A recombinant phage containing an *Eco*RI insert of 19 kilobase pairs (kbp) of normal human DNA, which included the c-*has/bas* proto-oncogene (6). The single 115-14 DNA-induced transformant, designated 134-51, was picked by the cloning cylinder procedure, cloned in soft agar, and its DNA was submitted to Southern blot analysis to determine whether it contained c-*has/bas* sequences. The results are depicted in Fig. 1. Whereas 115-14 DNA exhibited multiple copies of the c-*has/bas* gene, 134-51 DNA only contained one or few copies of this human gene. In fact, Southern blot analysis of 134-51 DNA digested with *Eco*RI and *Hin*dIII, two restriction endonucleases that cleave λbc-N DNA outside the c-*has/bas* sequences, indicated that 134-51 cells contain a single copy of this human oncogene (data not shown).

To determine whether this gene was responsible for the transformed phenotype of 134-51 cells or its presence was due

Abbreviations: kbp, kilobase pair(s); bp, base pair(s).

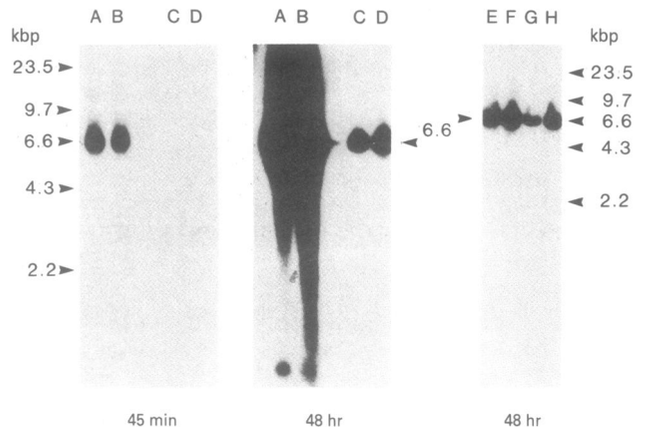

FIG. 1. Detection of c-*has/bas* sequences in the DNA of NIH/3T3 transformants. Lanes: A and B, independent proto-oncogene-induced transformants (115-14 and 136-41) containing 30–50 copies of the c-*has/bas* gene; C, T24 oncogene-induced transformant containing a single copy of the T24 oncogene; D, 134-51 cells, a transformant derived from 115-14 DNA containing the spontaneously activated c-*has/bas*; and E–H, independent second-cycle transformants derived from 134-51 DNA. Twenty micrograms of DNA from each of the above NIH/3T3 transformants was digested with the restriction endonuclease *Bam*HI, electrophoresed in a 0.7% horizontal agarose gel, blotted to a nitrocellulose sheet as described (17), and hybridized for 48 hr to 2×10^7 cpm of a ^{32}P-labeled probe prepared by nick-translation of the 3.0-kbp *Sac* I DNA fragment of pbc-N1, a pBR322 derivative containing the human c-*has/bas* proto-oncogene (12). Hybridized blots were exposed to Kodak XAR-5 film at −70°C in the presence of intensifier screens for either 45 min or 48 hr as indicated. Coelectrophoresed DNA fragments of *Hin*dIII-digested λc1857 DNA served as size standards (labeled in kbp).

to nonspecific cotransfection, we assayed the ability of 134-51 DNA to transform NIH/3T3 cells in an additional cycle of transfection. 134-51 DNA induced an average of 0.36 foci per microgram of donor DNA, an efficiency comparable to that obtained with DNAs containing a single copy of a dominant transforming gene. As shown in Fig. 1 (lanes E–H), each of the 134-51-induced NIH/3T3 transformants contained c-*has/bas* sequences. These results strongly suggest that one of the multiple copies of the c-*has/bas* proto-oncogene originally present in 115-14 DNA must have acquired transforming properties. Because 115-14 DNA did not induce transformation of NIH/3T3 cells in two additional transfection experiments, it is very likely that the malignant activation of the c-*has/bas* gene occurred during transfection of 134-51 cells.

A Single Point Mutation Responsible for the *in Vitro* Activation of the Human c-*has/bas* Proto-Oncogene. The c-*has/bas* gene present in 134-51 cells was molecularly cloned to unequivocally demonstrate its transforming properties and to determine the mechanism by which it became activated. For this purpose, 134-51 genomic DNA was digested with *Bam*HI and fractionated by sucrose gradient centrifugation. DNA fragments of 5.0–9.0 kbp were subsequently ligated to *Bam*HI-cleaved λ1059 DNA (20) at a 1:1 molar ratio. Ligated DNA was packaged into λ particles and the recombinant phages were selected by infection onto *Escherichia coli* Q359 permissive cells. Phages containing c-*has/bas* sequences were identified by standard filter hybridization techniques (21) by using the ^{32}P-labeled 3.0-kbp *Sac* I DNA fragment of the T24 oncogene (6, 7, 9) as a probe. A recombinant phage exhibiting a 6.6-kbp *Bam*HI DNA insert containing the activated c-*has/bas* gene was selected and tested for its ability to transform NIH/3T3 cells. This recombinant clone, designated λ9, exhibited a specific transforming activity of 8×10^3 focus-forming units per microgram of transfected phage DNA. These results established that 134-51 NIH/3T3 transformed cells harbored a transforming human c-*has/bas* gene.

It has been recently shown that a single point mutation within the first exon of the c-*has/bas* gene was responsible for its malignant activation in T24 human bladder carcinoma cells (11–14).

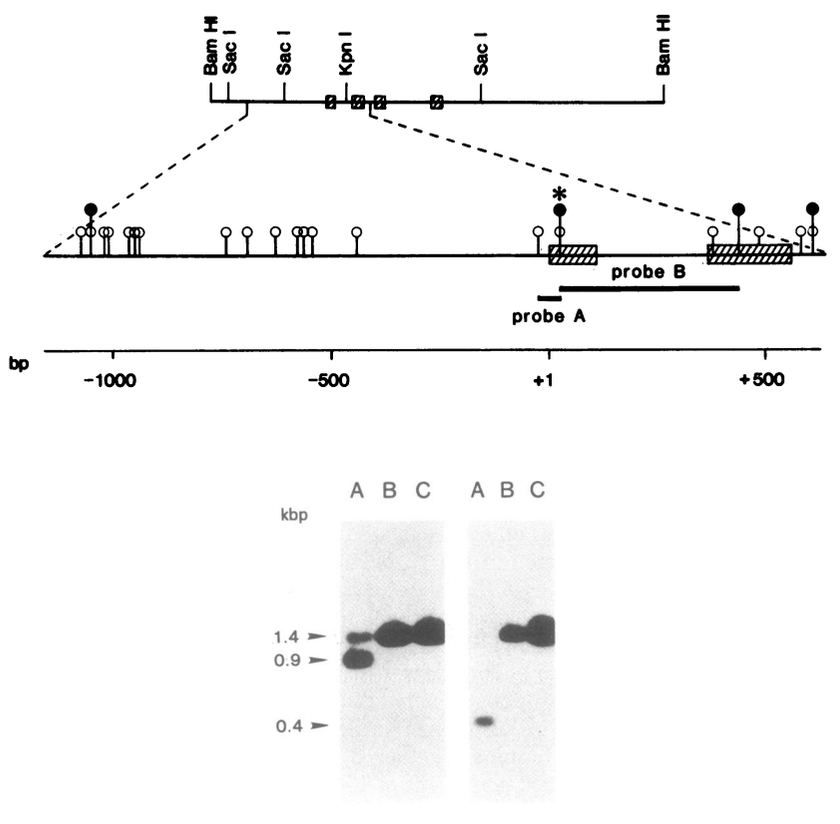

FIG. 2. The spontaneously activated c-*has/bas* oncogene has suffered a genetic alteration within the *Nae* I cleavage site located at positions +31 to +36 of the first exon of the c-*has/bas* proto-oncogene. (*Lower*) Lanes: A, λbc-N, a recombinant λCharon 4A phage containing a 19-kbp *Eco*RI insert of normal human fetal liver DNA that includes the c-*has/bas* proto-oncogene (6); B, λT24-15A, a recombinant λCharon 9A phage containing a 15-kbp *Eco*RI insert of T24 DNA that includes the T24 oncogene (8); and C, λ9, a recombinant λ1059 phage containing a 6.6-kbp *Bam*HI insert of human DNA present in 134-51 cells that includes the *in vitro* activated c-*has/bas* proto-oncogene. One microgram of each phage DNA was digested with the restriction endonuclease *Nae* I, electrophoresed in a 1.2% horizontal agarose gel, blotted to a nitrocellulose sheet, and hybridized to 5×10^6 cpm of either probe A (a 56-base pair bp) *Hpa* II DNA fragment expanding from positions −24 to +32 of the c-*has/bas* proto-oncogene) or probe B (a 412-bp *Nae* I DNA fragment expanding from positions +33 to +444 of the c-*has/bas* proto-oncogene). The size of each of the detected DNA fragments (indicated by arrows) was deduced by coelectrophoresing DNA fragments of *Hae* III-digested ΦX174 replicative form DNA. (*Upper*) The 6.6-kbp *Bam*HI fragment of human DNA containing the c-*has/bas* proto-oncogene. The hatched boxes represent the four proto-oncogene exons. ●, *Nae* I cleavage sites; *, the *Nae* I cleavage site not present in the T24 oncogene; and ○, *Hpa* II/*Msp* I cleavage sites.

The mutation, a G→T transversion, altered a G-C-C-G-G-C sequence specifically recognized by the restriction endonuclease *Nae* I, thus providing a simple biochemical marker for the identification of this oncogene. To examine whether a similar mutation may have occurred in the activated c-*has/bas* gene cloned from 134-51 NIH/3T3 transformed cells, we submitted it to digestion with *Nae* I. The resulting DNA fragments were separated by electrophoresis in an agarose gel and hybridized with two different DNA probes that specifically recognize this region of the c-*has/bas* gene. As shown in Fig. 2, the activated c-*has/bas* gene, like the T24 oncogene, generated a single 1.3-kbp *Nae* I DNA fragment. However, the normal gene exhibited the expected 0.9- and 0.4-kbp *Nae* I DNA fragments (11–14) in addition to residual amounts of uncleaved 1.3-kbp DNA. These findings indicate that the activated c-*has/bas* gene must have undergone a genetic alteration(s) at or very near the G→T transversion that conferred transforming properties to the T24 oncogene.

To determine whether this genetic alteration was responsible for the activation of the c-*has/bas* gene in 134-51 cells, we constructed a recombinant molecule that contained the entire normal c-*has/bas* proto-oncogene with the exception of a 693-bp *Bst*EII–*Kpn* I DNA region that was provided by the activated c-*has/bas* gene (Fig. 3). This 693-bp *Bst*EII–*Kpn* I DNA fragment contains the entire first exon, including the mutated G-C-C-G-G-C sequence. The chimeric clone was shown to transform NIH/3T3 cells with a specific activity of 1.4×10^4 focus-forming units per microgram of DNA, an efficiency comparable to those of the activated c-*has/bas* gene and the T24 oncogene.

The nucleotide sequence of the 693-bp *Bst*EII–*Kpn* I DNA fragment of the activated c-*has/bas* gene was next established and compared with the corresponding region of the nontransforming c-*has/bas* molecule (Fig. 3). As depicted in Fig. 4, we observed a single nucleotide difference, a G→A transition, located at position 35 of the first exon. These findings demonstrate that the human c-*has/bas* proto-oncogene can be spontaneously activated *in vitro* by a single point mutation within its coding sequences. More importantly, this *in vitro* G→A transition occurred at the *same* nucleotide position as the G→T transversion responsible for acquisition of malignant properties by an oncogene present in T24 human bladder carcinoma cells.

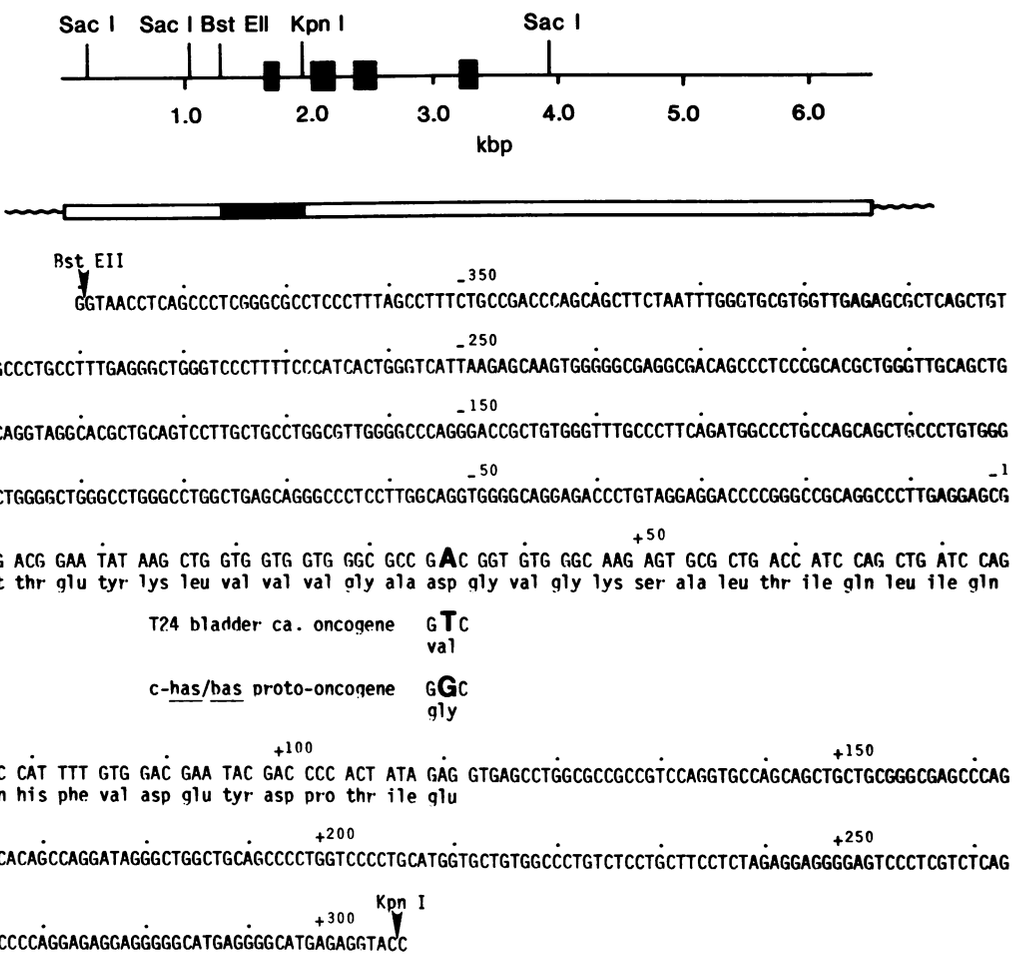

FIG. 3. A point mutation at position +35 is responsible for the spontaneous activation of the c-*has/bas* oncogene. Location of the region containing the genetic alteration(s) responsible for the activation of the c-*has/bas* gene was accomplished by constructing a chimeric clone in which the 693-bp *Bst*EII–*Kpn* I DNA fragment of pbc-N1, a plasmid containing the normal c-*has/bas* proto-oncogene (▭) (12), was substituted by the corresponding domain of the activated c-*has/bas* oncogene (■). This chimeric clone transformed NIH/3T3 cells with a specific activity of 1.4×10^4 focus-forming units per microgram of DNA. Comparative sequence analysis of the 693-bp *Bst*EII–*Kpn* I DNA fragment of the normal and activated c-*has/bas* genes revealed a single nucleotide difference, a G→A transition, at position +35. This nucleotide change results in the incorporation of aspartic acid as the 12th amino acid residue of the transforming p21 protein instead of the glycine residue present in the normal protein. For comparative purposes we included the sequence of the T24 oncogene in which a G→T transversion at the first two nucleotides of the GGC triplet coding for the 12th amino acid same position led to the incorporation of valine (11–14). In agreement with the sequences published by Taperowsky *et al.* (13) and by Capon *et al.* (14), we have observed that the 693-bp *Bst*EII–*Kpn* I DNA fragment of the T24 oncogene contains an additional nucleotide difference, a C instead of a T, at position −10 from the ATG initiator codon (22). The nucleotide sequence analysis was performed according to the procedures of Maxam and Gilbert (19).

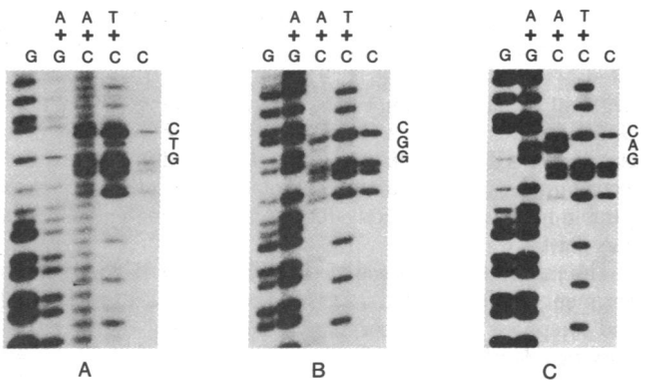

FIG. 4. DNA sequence analysis of nucleotides +20 to +40 of the coding sequences of T24 oncogene (*A*), normal c-*has/bas* proto-oncogene (*B*), and spontaneously activated c-*has/bas* gene (*C*). DNA was cleaved at the *Ava* I site located at position −26, labeled with [γ-^{32}P]-ATP, subjected to base-specific cleavage reactions, and analyzed on 0.3-mm thick, 8% polyacrylamide gels as described (19). The sequence of the crucial 12th codon (nucleotides +34 to +36) that has undergone the point mutation is indicated.

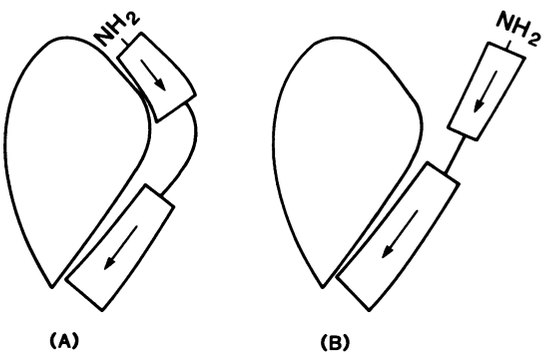

FIG. 6. Schematic diagram of the secondary structure of p21 proteins coded for by the normal c-*has/bas* proto-oncogene (A) and its transforming alleles (B). The two open boxes represent the two α-helical regions that approximately encompass amino acid residues 1–10 and 19–32 in the normal p21 protein and amino acid residues 1–13 and 17–32 in the transforming p21 proteins. The flexible coil region is represented by a solid line. The polarity of the molecule is indicated by the arrows. The closed area at the left of each drawing represents the core of the p21 molecule.

Substitution of Glycine by Aspartic Acid or Valine as the 12th Amino Acid Residue of Human p21 Has the Same Structural Consequences. Demonstration that the spontaneous activation of the human c-*has/bas* gene is mediated by a single point mutation within its coding sequences poses the basic question of how the substitution of glycine for aspartic acid as the 12th amino acid residue affects the structure of p21. For this purpose we analyzed the secondary structure of both normal and transforming p21 proteins by applying the method of Garnier *et al.* (23) to the deduced amino acid sequence of these proteins. In this analysis, assignment of antiparallel/parallel β strands was made according to procedures described by Lifson and Sanders (24). The program used has a parameter that determines the amount of bias towards α-helix and β-sheet structures in this region.

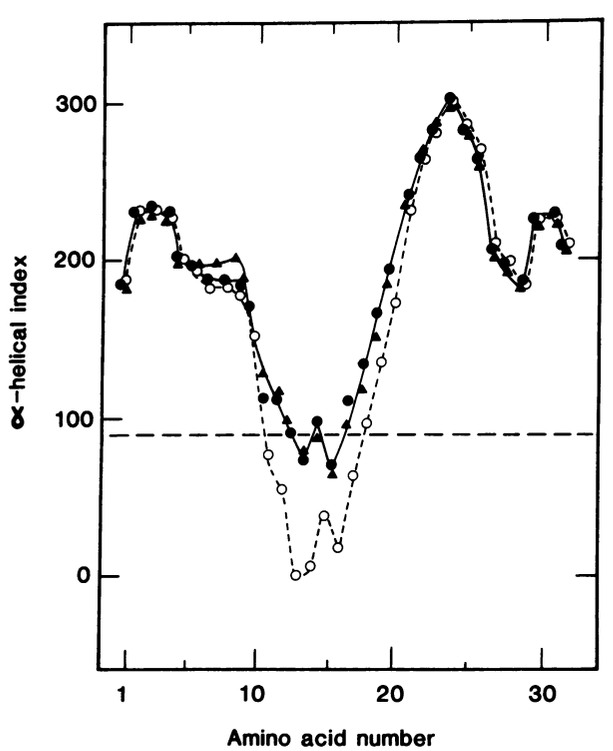

FIG. 5. α-Helical profiles of the first 32 amino acid residues of the amino-terminal regions of the p21 proteins encoded for by c-*has/bas* proto-oncogene (○), T24 oncogene (●), and spontaneously activated c-*has/bas* gene (▲). Protein secondary structure predictions were made according to the methods described by Garnier *et al.* (23) and Lifson and Sanders (24). The relative α-helical content is plotted against the amino acid residue number. The analysis shows the α-helical profile of the first 32 amino acids coded for by the first exon of each gene. The horizontal dotted line represents the arbitrary cutoff used in the Garnier's analysis as the limit for helical regions.

The analysis of the normal p21 protein indicates two helical regions. A short α helix of 10 amino acid residues (a strong stable helix requires about 8 residues, a little more than two complete turns) at the amino terminus is followed by a nonhelical region of 9 amino acids described in the Garnier analysis as a coil. This coiled region is followed by a stretch of 13 residues that elicit a strong helical response (Fig. 5). The combined pattern of structure as predicted by Garnier's method is two α helices separated by a coil region. We have used an arbitrary cutoff of 90 in the Garnier's analysis as the limit for helical regions. Substitution of glycine at position 12 by aspartic acid produced a significant change in the combined pattern, decreasing the length of the coil region and concomitantly strengthening the two flanking helices (Fig. 5). Thus, it is predicted that the p21 protein coded for by the *in vitro* activated c-*has/bas* gene contains an amino-terminal α helix of 13 instead of 10 amino acids, whereas the coil region is decreased to 3 amino acids and the other flanking helix is increased to 16 amino acids. As a consequence, the amino terminus domain of the transforming p21 protein will acquire a rigid configuration that will prevent it from folding into the core of the molecule, as is predicted to occur in its normal counterpart. Fig. 6 schematically depicts these structural features of both normal and transforming p21 proteins.

The p21 protein coded for by the T24 bladder carcinoma oncogene contains valine instead of aspartic acid at position 12 (11–14). Yet, computer analysis of the secondary structure of this protein predicts the same structure as that of the p21 protein coded for by the *in vitro* activated c-*has/bas* gene: two strong α-helical regions of 13 and 16 residues separated by a minor coiled region of 3 amino acids. In fact, mutation of any of the first two nucleotides of the GGC triplet coding for the 12th amino acid residue of the normal human p21 protein would lead to the incorporation of an amino acid that would cause similar struc-

tural alterations to those produced by either valine or aspartic acid. These observations, along with the transforming properties of the T24 oncogene and the *in vitro* activated c-*has/bas* gene described here, strongly suggest a direct correlation between specific changes in the secondary structure of the amino terminus domain of p21 and its ability to induce malignant transformation.

DISCUSSION

The present studies were initiated by the observation that a normal human proto-oncogene can spontaneously acquire transforming properties without the action of known carcinogenic agents. This finding came as a result of studies aimed at ascertaining the putative oncogenic properties of the c-*has/bas* proto-oncogene. Chang *et al.* have previously reported that this normal human gene can transform NIH/3T3 mouse cells when its coding sequences are placed under the control of retroviral regulatory elements (25). In studies to be reported elsewhere, we have extended these observations by showing that the c-*has/bas* proto-oncogene contains all of the necessary information to drive NIH/3T3 to malignancy. However, the combined effect of multiple (30–50) copies of this proto-oncogene is required to induce neoplastic transformation (unpublished data). As a consequence, DNA isolated from proto-oncogene-induced NIH/3T3 transformed cells failed to induce foci when tested in transfection assays. An exception to these results was the observation reported in the present studies in which one of the c-*has/bas* proto-oncogene copies present in an NIH/3T3 transformant spontaneously acquired malignant properties during the course of a transfection assay.

Molecular characterization of this *in vitro* activated oncogene allowed us to determine that a single point mutation was responsible for the acquisition of its transforming properties. A deoxyguanosine located at position 35 of the first exon of the c-*has/bas* proto-oncogene was changed into a deoxyadenosine in the transforming gene. Substitution of the *same* nucleotide has been recently found to be responsible for the activation of this gene in T24 human bladder carcinoma cells (11–14). These findings indicate that mutations affecting this particular nucleotide will result in the malignant activation of the c-*has/bas* proto-oncogene. It should be noted that the two mutations observed, a G→T transversion (T24 oncogene; refs. 11–14) and a G→A transition (present studies) have different effects on the coding properties of these oncogenes. Whereas the spontaneously activated c-*has/bas* gene would direct the incorporation of aspartic acid as the 12th amino acid residue of its translational product p21, the T24 oncogene product would contain valine instead. Considering the different biochemical properties of these two amino acids, it is quite remarkable that substitution of glycine by either of these two amino acid residues leads to the same biological effect, the malignant transformation of NIH/3T3 cells.

An explanation of these findings has been provided by computer analysis (23, 24) of the predicted secondary structure of p21 proteins containing different amino acid residues at position 12. Substitution of the normal glycine by any amino acid containing a side chain will cause the same structural alteration: disappearance of a flexible hinge region that allows the amino-terminal domain of the normal p21 protein to fold into the central core of the molecule. Understanding how this structural change affects the cellular role of p21 is hampered by our limited knowledge of its biochemical properties. However, these studies suggest that substitution of either, or both, deoxyguanosine residues present at positions 34 and 35 of the first exon of the human c-*has/bas* proto-oncogene will lead to its malignant activation.

Demonstration of the important role of amino acid residue 12 on the biological activity of the c-*has/bas* gene product does not eliminate the possibility that other structural changes may also confer transforming properties to this protein. More detailed computer analysis of the secondary and tertiary structure of p21 in combination with studies involving site-directed mutagenesis of the c-*has/bas* proto-oncogene should lead to a better understanding of the role of this gene in oncogenesis.

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1. Ellis, R. W., DeFeo, D., Shih, T. Y., Gonda, M. A., Young, H. A., Tsuchida, N., Lowy, D. R. & Scolnick, E. M. (1981) *Nature (London)* **292,** 506–511.
2. Cooper, G. M. (1982) *Science* **217,** 801–806.
3. Andersen, P. R., Devare, S. G., Tronick, S. R., Ellis, R. W., Aaronson, S. A. & Scolnick, E. M. (1981) *Cell* **26,** 129–134.
4. Der, C. J., Krontiris, T. G. & Cooper, G. M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3637–3640.
5. Parada, L. F., Tabin, C. F., Shih, C. & Weinberg, R. A. (1982) *Nature (London)* **297,** 474–478.
6. Santos, E., Tronick, S. R., Aaronson, S. A., Pulciani, S. & Barbacid, M. (1982) *Nature (London)* **298,** 343–347.
7. Goldfarb, M., Shimizu, K., Perucho, M. & Wigler, M. (1982) *Nature (London)* **296,** 404–409.
8. Pulciani, S., Santos, E., Lauver, A. V., Long, L. K., Robbins, K. C. & Barbacid, M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2845–2849.
9. Shih, C. & Weinberg, R. A. (1982) *Cell* **29,** 161–169.
10. Chang, E. H., Gonda, M. A., Ellis, R. W., Scolnick, E. M. & Lowy, D. R. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4848–4852.
11. Tabin, C. J., Bradley, S. M., Bargmann, C. I., Weinberg, R. A., Papageorge, A. G., Scolnick, E. M., Dhar, R., Lowy, D. R. & Chang, E. H. (1982) *Nature (London)* **300,** 143–149.
12. Reddy, E. P., Reynolds, R. K., Santos, E. & Barbacid, M. (1982) *Nature (London)* **300,** 149–152.
13. Taparowsky, E., Suard, Y., Fasano, O., Shimizu, K., Goldfarb, M. & Wigler, M. (1982) *Nature (London)* **300,** 762–765.
14. Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H. & Goeddel, D. V. (1983) *Nature (London)* **302,** 33–37.
15. Graham, F. L. & van der Erb, A. J. (1973) *J. Virol.* **52,** 456–463.
16. Wigler, M., Silverstein, S., Lee, L. S., Pellicer, A., Cheng, Y. & Axel, R. (1977) *Cell* **11,** 223–232.
17. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
18. Maniatis, T., Fritsch, E. F. & Sambrook, J., eds. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
19. Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 560–564.
20. Karn, J., Brenner, S., Barnett, L. & Cesareni, G. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5172–5176.
21. Benton, W. D. & Davis, R. W. (1977) *Science* **19,** 180–181.
22. Reddy, E. P. (1983) *Science,* in press.
23. Garnier, J., Osguthorpe, D. T. & Robson, B. J. (1970) *J. Mol. Biol.* **120,** 97–120.
24. Lifson, S. & Sanders, C. (1979) *Nature (London)* **282,** 109–111.
25. Chang, E. H., Furth, M. E., Scolnick, E. M. & Lowy, D. R. (1982) *Nature (London)* **297,** 479–483.