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Ha-ras in normal and tumoral tissues: structure, function and regulation

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The *c-Ha-ras1* gene belongs to an eucaryotic ubiquitous gene family which codes important molecules involved in the transduction of mitogenic signals and of cellular differentiation. The *c-Ha-ras1* estructure, in four coding exons and a non-coding 5'exon, is highly preserved throughout evolution. This gene, which generates a 1.4 Kb transcript, is expressed in practically all the cell lineages with a tissue-specific pattern. The coded protein, of 189 amino acids and 21 kDa, is a small protein that binds guanine nucleotides (GTP/GDP), is associated with the plasma membrane and possesses low intrinsic GTPase activity. p21ras functions as a molecular switch active when GTP is bound to it and inactive in the GDP-bound form. Its activity is very tightly controlled in the cell by various positive and negative control mechanisms. The *ras* gene family is the most frequently involved in the development of human and animal tumors. Qualitative (point mutations) and quantitative (over expression) mechanisms of oncogenic activation have been described. The possible relation between determined human *Ha-ras1* alleles and the predisposition to cancer has been suggested as well.

Key words: *Ha-ras*, p21ras, Oncogene.

The *Ha-ras* (*c-Ha-ras1*) gene belongs to an eucaryotic ubiquitous gene family, very conserved throughout evolution. All the members of this family code proteins associated with the plasma membrane that bind to guanine nucleotides (GTP/GDP) and play a basic role in the transmission of signals involved in cellular growth and differentiation (5, 69).

In the mammalian genome that family includes, besides *c-Ha-ras1*, two other genes: *c-Ki-ras2* and *N-ras* (27, 29, 31, 47, 85, 99, 104). On the other hand, processed pseudo genes from each of these genes

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have been identified in humans and mice, *c-Ha-ras2*, *c-Ki-ras1*, and *N-ras1.2ep* and *N-ras6.1ep* respectively (27, 31, 44, 74, 75). Genes and pseudogenes of the *ras* family are dispersed in different chromosomes in distinct species. Thus, in humans, *c-Ha-ras1*, *c-ki-ras2* and *N-ras* are located in the short arms of chromosomes 11,12 and 1 respectively. The human *c-Ha-ras2* pseudogene is found in chromosome X and the *c-Ki-ras1* in the 6. In mice, *c-Ha-ras1* has been mapped in chromosome 7, whereas *c-Ki-ras2* and *N-ras* have been assigned to chromosomes 6 and 3 respectively. Finally, in rats, *c-Ha-ras1* has been located in chromosome 1, *c-Ki-ras2* in chromosome 4 and *N-ras* in chromosome 2; the *c-Ha-ras2* pseudogene is found in chromosome X (53, 58, 82, 83, 111).

The *ras* genes have also been identified in yeasts, *Drosophila*, *Caenerhabditis elegans* and *Dyctiostelium discoideum* (36).

The *c-Ha-ras1* cellular gene was originally described as the oncogene of Harvey murine sarcoma retrovirus (HaMuSV). *c-Ki-ras2* is, likewise, the cellular homologue of the transforming gene of Kirsten murine sarcoma virus (KiMuSV). In both cases, the viral genes originated by transduction from a rat cellular gene (20, 27, 29, 31). The third family member, *N-ras*, was characterized as transforming gene homologous to the other *ras* genes in a human cellular line of neuroblastoma, and it has not yet associated itself to any retroviruses (20, 29-31, 33, 104).

The gene family to which *c-Ha-ras1* belongs is a part, in turn, of a superfamily of genes which code a type of small proteins that bind to GTP/GDP, structurally related (table I) (40).

The Ras superfamily of proteins is estimated to be composed at present by over 50 members. All of them are characterized by having an oscillating 20 - 29 kDa size, by the presence of consensus motifs for GTP-binding proteins, and by the existence of other sequence motifs characteristic of each subfamily. The Ras superfamily can be subdivided into five subfamilies. The three main ones and the most related ones between themselves are those of Ras, Rho and Rab, each one of which includes a variable number of related proteins.

Table I. *Families of mammalian GTP/GDP-binding proteins (59, 115).*

- The elongation factors of protein biosynthesis.
- Subunits of the signal recognition particle (SRP) and its receptor.
- The ADP-ribosilation factor family (ARF)
- The α subunits of heterotrimeric G-proteins involved in signal transduction.
- The products of the *ras* gene superfamily.
  Subfamilies:
  - Ras and related family: Ha-ras, Ki-ras (A and B), N-ras, rap1A6 (also smg p21A and Krev-1), rap1B (also smg p21B), rap2A, rap2B, ral A, ral B, R-ras, TC21.
  - Rho and related family: rhoA, rhoB, rhoC, TC10, rac 1 (also TC25), rac 2 (also EN-7), G25K (also Gp and CDC42Hs).
  - Rab and related subfamily: rab1A (also ypt1), rab1B, rab2, rab3A (also smg p25A), rab3B, rab3C, rab4, rab5, rab6, rab7 (also BRL-ras), rab8 (also MEL), rab 9, rab10, rab11 (also YL8 and 24KG), smg p25B, smg p25C, ram.
  - Ran and related subfamily: TC4, ran.
  - Arf and related subfamily: arf1, arf2, arf3, arf4, arf5, arf6.

A fourth subfamily, more distant, is made up of Arf and related proteins. Finally, the TC4 and Ran proteins constitute the fifth subfamily designated as that of Ran related proteins. This subdivision of the Ras superfamily into subfamilies has a structural basis rather than a functional one and has been carried out basically from the alignments of the sequence. Thus, the alignment of a subfamily member with another member of another subfamily reveals about 30 % of sequence identity, whereas this value is generally 40 % as minimum between two sequences within the same subfamily. Proceeding in this way a phylogenetic three has been made out from one common ancestral gene from which the members of the superfamily would have derived, probably by genic duplication (62, 63, 81, 117).

1. *c-Ha-ras1* structure

In mammals, *c-Ha-ras1* comprises four coding exons (13, 54, 74, 105, 113). This structure is common to the other *ras* family members, with which it shares homologous sequences and divergent regions. Nevertheless, *c-Ki-ras2* presents the peculiarity of possessing two alternative fourth coding exons (exons IVA and IVB), that generate two isomorphic proteins (14, 74, 105), and the rat *N-ras* gene, unlike human and murine genes (44, 113), possesses two non-translated additional exons (120). On the other hand, the three *ras* genes share, in mammals, an additional 5' non coding exon (exon 0) located immediately downstream the promoters (15, 57, 74).

The splicing junctions of *ras* genes match with precision that in each of them the corresponding exon codes the same part of the protein, suggesting again a common origin from one ancestral gene. Nevertheless, introns differ in size and sequence and, therefore, *ras* genes present different degrees of complexity. For instance, the coding sequences of human *c-Ha-ras1* span 3 Kb approximately, whereas those of *N-ras* occupy 7 kb and those of *c-Ki-ras2* comprise more than 35 Kb (5, 69). The *c-Ha-ras1* gene structure is shown in figure 1.

The general features of the promoters are also common in the three *ras* genes. In all of them, those regions lack the TATA and CAAT motifs, frequent in other eucaryotic genes. Instead they contain multiple transcriptional start sites and a high content in GC boxes, presumably involved in the binding of the transcription factor Sp1. This latter trait is proper to the housekeeping genes, which express in a constitutive way in the cell (54, 55).

The regulatory elements of human, rat and mouse *c-Ha-ras1* genes have been analyzed in detail. Thus, a region of about 350 pb that includes exon 0 has been identified, significantly well conserved between these species. This region, which comprises about 175 pb upstream and about 25 pb downstream the exon 0 is rich in GC and seems to be important for the control of the *c-Ha-ras1* expression. On the other hand, there exists a segment of conserved sequences in the 3' half of the first intron which has also been involved in the aforesaid control (23, 51). Similar characteristics have been found in the *c-Ki-ras2* (59) and *N-ras* (57) promoters. In addition these 5' regulatory elements, two other elements have been identified in the human *c-Ha-ras1* gene, located at the 3' end, which could contribute to the regulation of its expression (69). One is a region located downstream of the polyadenylation signal, which contains a number of variable tandem repeats (VTR) of a 28 pb element (13, 49). This region seems to present a weak enhancer activity, a certain minimum number of the repetitions being necessary for a complete activity to be obtained (114). The other 3' regulatory element is a conserved alternative exon

between exons 3 and 4 (16). This exon is 82 pb long and can code 20 amino acids before reaching a stop codon in the same reading frame. As the first three exons encode 150 amino acids, an mRNA containing this exon would code a potential product of 170 amino acids and 19 kDa (p19), different in its carboxy-terminal end from the functional protein p21 which include the amino acids corresponding to the fourth exon instead of those from the alternative exon (17). Nevertheless, mature transcripts containing this exon are scarce, due probably to their poor stability and/or to a defective processing, and they do not seem to have any biological activity. This alternative exon would constitute a regulatory element, being responsible not only for the basal level of gene expression, together with the promoter and enhancer elements, but also of an important negative modulatory activity. Thus point mutations in this region have been described which increase the expression and the transforming activity of the *c-Ha-ras1* oncogene -see section 5.2.- (16).

As previously quoted, in the normal human and rodent genome, besides the cellular *c-Ha-ras1* gene, there exists the *c-*

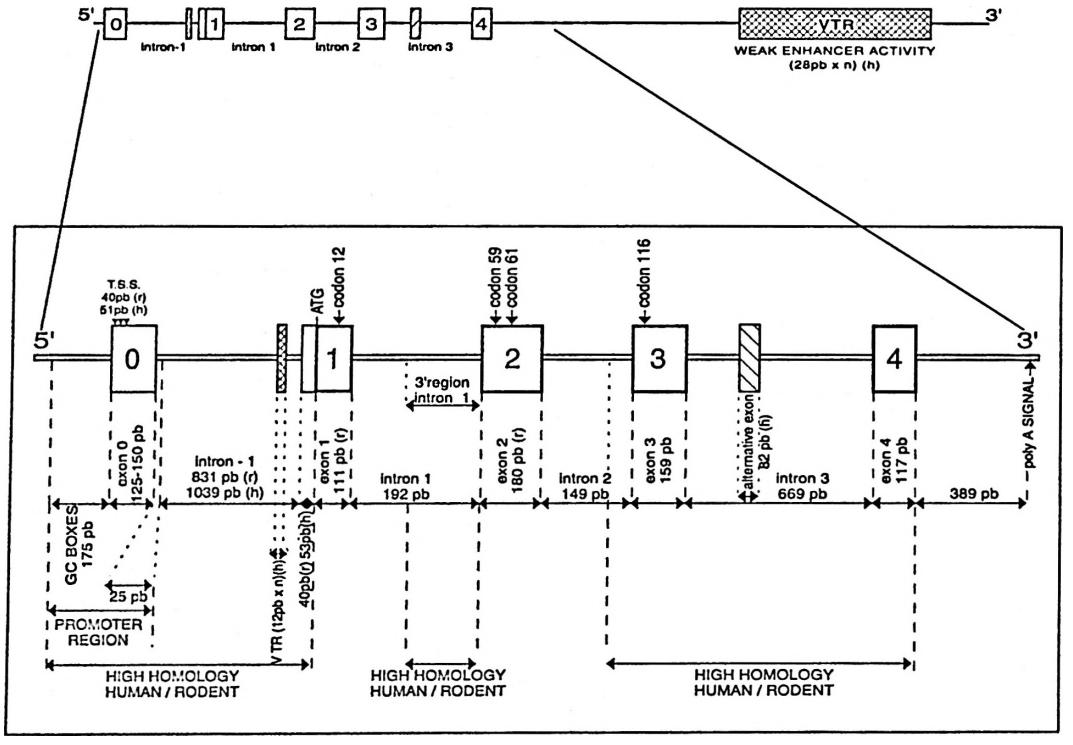

Fig. 1. *Schematic representation of the human and rat* c-Ha-ras1 *structure.*
The closed boxes represent translated sequences and the open ones the un-translated exon sequences. The hatched boxes indicate polymorphic regions of tandem repeats (VTR) described in the human gene. Several transcriptional start sites (T.S.S.) located at the 5' end of the exon 0, translation initiation codon (ATG) and the polyA addition signal, are indicated. Likewise, the most frequently altered codons by point mutations in *c-Ha-ras1* oncogene are shown. The regulatory elements of *c-Ha-ras1* and regions highly conserved between the human and rodent gene are also detailed (for more details, see in text). (h): human, (r): rat.

Ha-ras2 processed pseudogene, colinear with the viral *v-Ha-ras* gene (75, 96). In Southern blot hybridization analysis using as probe a fragment of the aforesaid viral gene it is possible to identify both types of homologous sequences (see section 5.1, fig. 5). Thus, in the Sprague-Dawley rat, the hybridization of genomic DNA digested with the restriction enzymes *Eco*RI, *Pst*I, *Bam*HI and *Xba*I

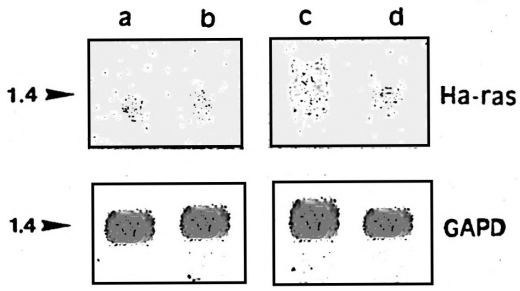

Fig. 2. *Expression of* c-Ha-ras1 *gene in liver and experimental mammary adenocarcinomas from Sprague-Dawley rats.*

Northern blot of total RNA from liver (lines a, b) and DMBA-induced mammary adenocarcinomas from female adult Sprague-Dawley rats (lines c, d) hybridized to a viral *v-Ha-ras* probe. Methods: The RNAs were extracted by the method of CHOMCZYNSKI and SACCHI (22) and were analyzed according to the standard procedures (98). RNA samples (25 µg) were electrophoresed on 1.1 % agarose gels containing 2.2 M formaldehyde, blotted by capillarity onto a nylon filter, UV-crosslinked and consecutively hybridized to an *Eco*RI-*Pst*I fragment of a *v-Ha-ras* clone (30) and a *Psf*I fragment of a cDNA clone of human *glyceraldehyde-3-phosphate-dehydrogenase* (*GAPD*) gene (116), both random primed -α^{32}P-dCTP- labeled. The hybridizations were for 16 hours at 42 °C in a solution which contained 50 % deionized formamide/5 x SSC (0.75 M NaCl, 0.075 M sodium citrate, pH 7)/50 mM Na_2HPO_4 pH 6.5/5 x Denhardt (0.2 % ficoll, 0.2 % polyvinylpyrrolidone), 0.2 % BSA/0.025 mg/ml tRNA/0.02 mg/ml poly A/0.1 mg/ml salmon sperm DNA. Filters were washed 4 times for a total of 40 minutes at room temperature in 2 x SSC, 0.1 % SDS and for 1 hour at +50 °C in 0.2 x SSC, 0.1 % SDS. Subsequently they were exposed to autoradiography with intensifying screens. The autoradiographic signals were digitalized and quantified by densitometry. The length of the detected transcript, expressed in Kb, was estimated in relation to the RNAr 28S and 18S markers.

generated fragments of 23, 1.1, 14 and 5.4 Kb respectively which corresponded to the *c-Ha-ras1* gene. Likewise, others additional fragments of 17, 1.6 and 7.2 Kb respectively resulted from the hybridization with the *c-Ha-ras2* pseudogene.

c-Ha-ras2 is found surrounded by several direct repeats which were probably generated by recombination during the integration process. The absence of transcription initiation signals, RNA cap sites and ATG translation initiation codons indicate that *c-Ha-ras2* is biologically silent. Furthermore, *c-Ha-ras2* contains multiple mutations with respect to *c-Ha-ras1* as well as one of the classic activating mutation of the *ras* genes (AGT at codon 12 of the gene which codes the human p21 protein) (27, 67, 75, 96). Its possible role in carcinogenesis will be discussed in section 5.

2. Expression of *c-Ha-ras1*

Figure 2 shows the result of a Northern blot analysis of total RNA from Sprague Dawley rats hybridized with a *v-Ha-ras* viral probe. A single species of about 1.4 kb *c-Ha-ras1* mRNA is observed in it. This result is generally in agreement with those described by other authors (23, 89, 114, 122). In mouse cells, a kind of additional polyadenylate RNA of 5 Kb has been described (32). The fact that the coding sequences of the gene is approximately 0.65 Kb in length means that, in any case, the mRNA contains a coding capacity much greater than the necessary one for the translated protein. The function of the 5' and 3' untranslated sequences is not completely known and it is thought that they are important in the regulation of expression and it is possible that they might influence the efficiency of mRNA translation (32).

In mammals, *c-Ha-ras1*, and generally all ras genes, are expressed at low levels in practically all cell lineages, although some

differences have been detected in expression throughout pre- and postnatal development (4, 66, 69). An instance of the low expression of this gene is shown in figure 2, where the mRNA levels of *c-Ha-ras1* can be seen, in comparison with those of the transcript control *glyceraldehyde-3-phosphate-dehydrogenase (GAPD)* in rat liver (lines a, b) and in DMBA-induced rat mammary tumors (lanes c, d).

Unlike other protooncogenes, *ras* genes are expressed throughout the development of the mouse embryo with a tissue-specific pattern. Likewise, some adult tissues preferentially express one type or other of ras gene (*c-Ha-ras1* in skin and skeletal muscle; *c-Ki-ras2* in intestine and thymus; *N-ras* in testicle and thymus) (66). Such a pattern of expression suggests that at least one of the three *ras* genes is expressed in all cell types (4, 69).

Due to the important role that *c-Ha-ras1* plays in the normal cellular growth during embryogenesis and cellular proliferation, its expression is found closely controlled in the cell. In actively proliferating tissues such as the liver in regeneration there has been found an increased expression (41). Increases of expression in human and experimental tumoral tissues can also be found (Section 5.2). For instance, in the Northern blot analysis shown in figure 2, the densitometric signal normalization of the *c-Ha-ras* mRNA obtained in a determined lane with the corresponding to the GAPD control transcript in the same lane, made it possible to establish in greater relative levels of the protooncogene in the tumoral samples than in those of liver.

Nevertheless the increase levels of *ras* expression do not always correlate with cellular proliferation. Thus, *ras* genes seem to play a dual role in cellular proliferation and in certain specific functions of terminally differentiated cells (5, 48, 69).

3. Ras proteins

c-Ha-ras1 codes for a 189 amino acid protein highly related to the proteins coded by the other functional *ras* genes. All of them are generically known as p21 due to their size of 21 kDa (48). In mammals there exist four Ras proteins because *c-Ki-ras2* produces, by alternative splicing, an additional protein (Ki-RasB) of 188 amino acids (14, 74).

The p21 proteins are characterized by their capacity to bind guanine nucleotides (GDP and GTP) with high affinity (of the order of 10^{11} to 10^{12} M^{-1}), to possess a low rate of dissociation and an intrinsic GTPase activity which is very small in relation to other G proteins. Their function is a key factor in the intracellular signal transduction acting as molecular switches, active in their form bound to GTP and inactive in the GDP-bound form (101, 125). The action mechanism of such a molecular switches base on the equilibrium that exists in the cell between both forms. Most p21ras molecules are found in their inactive state, whose conformation makes the GDP binding possible. The normal p21ras proteins remain in that state until they receive a stimulus from another protein (e.g. activated tyrosine kinase receptors). Such a stimulus will result in an exchange of GDP for GTP followed by a conformational change of the p21ras protein to its active state, where it can interact with potential effector molecules "downstream" to transmit the necessary signals for proliferation and differentiation. Once such an interaction has taken place, the p21ras proteins must be immediately deactivated. This can be done by their intrinsic GTPase activity, which catalyzes the hydrolysis of GTP reverting the protein to the inactive GDP-bound state (5, 48) (fig. 4).

3.1. Primary structure and function

The protein coded by *c-Ha-ras1* presents a high homology of sequence (about 90 %) with the other p21 proteins, which means that all of them possess very similar structural and functional characteristics.

Ras proteins can be divided into three regions (fig. 3) (68, 69, 117):

1) Catalytic domain: it is formed by the 165 amino-terminal amino acids of the protein and it is found very conserved in the three mammalian *ras* genes (more than 90 % homologous). This domain is composed of a central core of two β-sheets surrounded by peripherally located series of α helices and loops. The guanine nucleotides bind in a pocket formed by various segments of the catalytic domain, so that mutation of any of these segments can drastically reduce nucleotide binding. The catalytic domain contains, besides the necessary sequence motifs for the interaction with the nucleotide, the GTPase activity, which yields the slow hydrolysis of the bound GTP converting it into GDP which remains complex with the protein (101, 102). This GTPase activity together with the intrinsic dissociation rate of the GTP control the activation state of the protein. Finally, in this domain the effector region is also found, involved in the interaction between p21 and their cellular or effector target protein. This interaction is possible thanks to the conformational change that p21 undergoes when it binds to GTP (72).

2) Heterogeneous region: it is the one comprised between the 165 and 185 amino acids of the protein. It is completely different in the four p21 proteins but it is highly conserved in each one of them between humans and rodents. The function of the amino acids of the N-terminal end of this region is unknown, its importance for structural integrity of Ras or for its effector function not having been found. The amino acids of the C-terminal

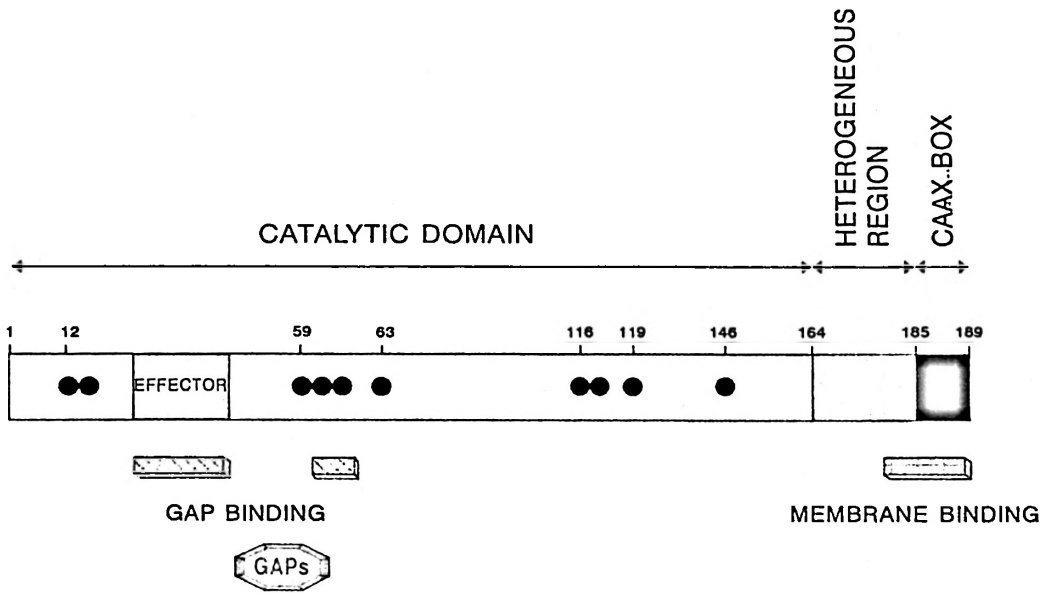

Fig. 3. Linear representation of the structural and functional domains of mammalian p21ras *proteins.* Amino terminaus appears at the left of the graph and the carboxy terminus on the right. Regions required for effector function, GAP proteins interaction and membrane binding are indicated. Black dots represent amino acids at which missense mutations enhance the transforming potential of p21.

end instead, probably participate in mediating the suitable subcellular localization of the protein as palmitoylated cysteines that contain 3 out of the 4 p21 proteins in this region increase membrane affinity. The number and localization of those cysteines vary among the different proteins. The heterogeneous region coded by the 4B exon of *c-Ki-ras2* contains six lysines instead of palmitoylated cysteines but these residues seem to act in the same way. The characteristics of the heterogeneous region suggest that it could be associated with different functions in the distinct Ras proteins.

3) CAAX box: it is made up of the last four amino acids of the cytosolic primary translation products and it is the one responsible for anchoring the protein to the plasma membrane, necessary for its

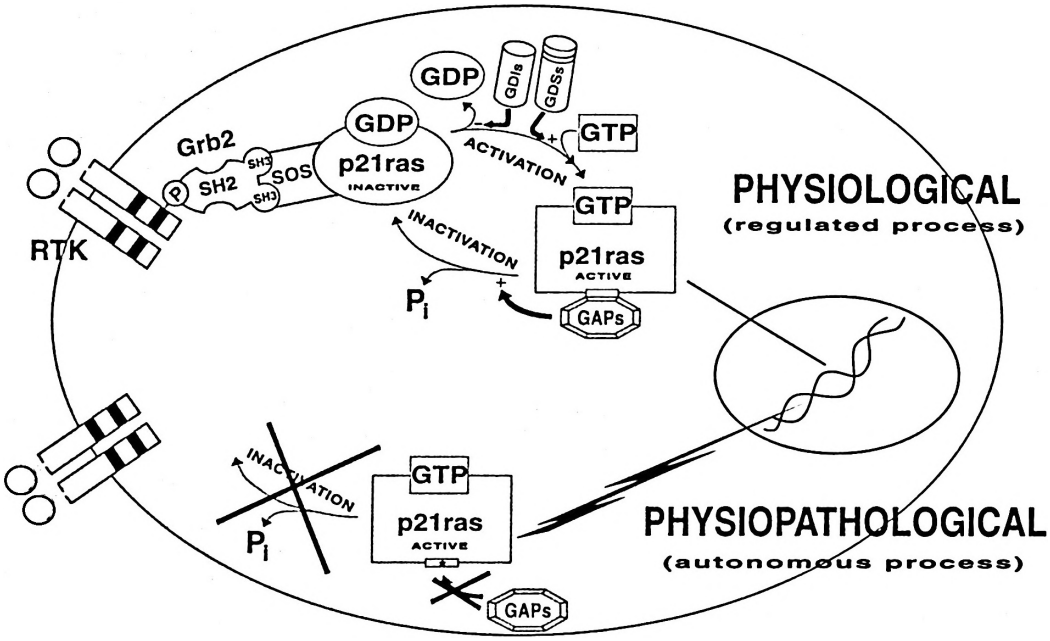

Fig. 4. Action mechanism pattern of normal and transforming p21ras *proteins.*
Ras proteins exist in equilibrium between an active and an inactive form. Most of the *p21ras* molecules of a cell exist in their inactive state, the conformation of which allows binding of GDP. Normal *p21ras* proteins stay in that state until they receive a stimulus from other protein (for instance, activated tyrosine kinase receptors) Such a stimulus would result in the exchange of GDP for GTP followed by a conformational change of the *p21ras* protein to its active state, in which it can interact with putative effector molecules. Once the interaction has taken place, the *p21ras* proteins ought to be immediately deactivated. This can be accomplished by their intrinsic GTPase activity, which catalyzes the GTP hydrolysis, returning the active Ras protein to the inactive GDP-bound state. Mutations which confer transforming properties to *ras* genes revert the normal equilibrium between the active and inactive forms. Stabilization of p21ras proteins in their active state would cause a continuous flow of signal transduction, which would result in malignant transformation. Theoretically, this process can be achieved by mutations that inhibit the intrinsic rate of the GTPase activity of Ras proteins, increasing the exchange between GDP and GTP, or inducing a conformational change that does not require binding of guanine nucleotides. High levels of normal protein can also produce enough molecules in their GTP-bound active state to induce malignant transformation without affecting the equilibrium between the active and inactive forms, which is characteristic of normal proteins.

biological activity. C is a cysteine common to all Ras proteins, A tends to be an aliphatic -nonpolar- amino acid, and X may be one of several amino acids. These four amino acids undergo posttranslational modifications consisting in farnesylation of Cys 186, proteolytic cleavage of the three carboxy terminal amino acids and carboxymethylation of this cysteine. These modifications, together with the reversible palmitoylation of cysteines located in the heterogeneous region immediately upstream the farnesylated cysteine, increase the hydrofobicity of the protein and results in its association with the inner face of the plasma membrane. In this way, these modifications promote the specific interaction of Ras protein with regulatory proteins and other components required for the Ras mediation in signal transmission. The modifications that allow the Ras translocation to the inner face of the plasma membrane are necessary for the Ras transforming activity (45).

Numerous studies based on the use of deletion mutants and neutralizing monoclonal antibodies as well as crystallographic analysis have made it possible to assign the different biochemical properties of Ras proteins to specific domains within these molecules. Thus mutationally activated (transforming) p21 proteins became associated with alterations in amino acids which decrease the GTPase activity and/or increase the GDP/GTP exchange rate, producing an abnormally high quantity of active GTP-bound protein. The most commonly isolated *ras* oncogenes have a point mutation in codons 12, 13 or 61. Substitution of the Gly12 or the Gln61 of the normal protein by a wide range of amino acids reduces its GTPase intrinsic activity, renders it resistant to the regulation by the GTPase-accelerating activity of GAP and neurofibromin proteins and makes the p21 transforming (fig. 4). Ras can also be activated by mutation of Ala59 to Thr or Glu63 to Lys or His. The Thr59 mutation is particularly interesting because this residue is autophosphorylated in those *ras* oncogenes in which it has identified. This reaction represents the transfer of the γ-phosphate of bound GTP to the hydroxyl group of Thr59 and its discovery was the first indication that the Ras proteins might have a GTPase activity that would regulate Ras and other guanine nucleotides-binding proteins. This mutation, present in *v-Ha-ras* and *v-Ki-ras*, which possess also the mutation in codon 12 reduces the GTPase intrinsic activity and the sensitivity to GAP, increases the GDP/GTP exchange and activates transformation. On the other hand, the Ras activation that results from the mutation of residues 116, 119 or 146 is associated to a substantial increase in the intrinsic guanine nucleotide exchange rate. This implies a significant reduction of the GDP and GTP affinity for Ras, which favors the formation of the active complex due to the greater availability of GTP *in vivo*. Deletion of residues 152-165 completely eliminates guanine nucleotides binding and transforming activity. Finally, mutations in the 30-42 residues, of a hydrophylic nature and located in the outer surface of the molecule, do not seem to alter the Ras p21 biochemical properties, which suggests that such residues are engaged in their effector activity (4, 46, 66, 68, 121).

3.2. Regulation of the activity

The half-life of a p21 protein is greater than 24 hours, suggesting that its regulation is carried out mainly at the level of protein activity. The p21 activity can be regulated by modulation of the proportion of GTP-bound to p21 or the membrane association of the protein. There exist no clear evidences of the association of various states of cellular growth to

changes in the protein interaction with the membrane, in spite of the fact that the half-life of the palmitate bound to the cysteines of the p21 heterogeneous region is short (about 20 min). Contrariwise, numerous works have shown the regulation of p21 activity by the concentration of p21-GTP. Thus, the interconversion between the GDP-bound inactive p21-GTP form and the active GTP-bound form is found tightly controlled at the cell. There exist two types of regulatory proteins: those that stimulate the low GTPase intrinsic activity of Ras proteins and those that mediate in the exchange between GDP and GTP (fig. 4). The deregulation of the function of one of these proteins can provoke the constitutive elevation of Ras-GTP in the absence of activating mutations (48, 68, 69, 126).

The first activating factor of Ras intrinsic GTPase activity ("GTPase activating protein" -GAP-) identified in vertebrates was a 120 kDa protein (p120GAP) (26, 40, 115). Two proteins related to p120GAP, IRA1 and IRA2, have been identified in yeasts. All of them accelerate the hydrolysis rate of bound GTP of p21ras-GTP in more than two orders of magnitude. The powerful negative regulatory action of p120GAP is brought about after its specific union, through its carboxi terminal catalytic domain, to the p21 effector domain. Besides this regulatory action, p120GAP would have a second function as a downstream effector of Ras activity (60). The present model suggests that the p21ras-GTP binding to the catalytic domain of p120GAP provokes a conformational change of this protein which exposes its amino terminal region to putative downstream targets allowing interaction with them and transmission of the Ras signal up to the nucleus. In this amino terminal region of p120GAP, there exist two SH2 and one SH3 domains (homologous to the second and third non catalytic domains of the Src oncogenic proteins) which would be the ones in charge of mediating protein-protein interactions. It is interesting to point out that p120GAP cannot regulate negatively other members of the Ras superfamily and that, as it was previously indicated, Ras proteins activated by point mutations at residues 12 or 61 are resistant to the GTPase accelerating catalytic activity of p120GAP (40, 72).

A second protein with GAP activity is neurofibromin, coded by the NF-1 tumor suppressor gene associated with neurofibromatosis type 1. This protein, of 250 kDa, shares sequence homology and substrate specificity with the p120GAP C-terminal catalytic domain. Like this one, NF-1 stimulates the hydrolysis of GTP bound to Ras causing its inactivation. Furthermore it also seems to play a second role as downstream effector of Ras activity (63, 68, 69).

In vivo, the spontaneous dissociation rate of bound GDP from p21 protein is very low but there exist factors which positively regulate Ras by stimulating GDP dissociation and GDP/GTP exchange ("guanine nucleotide dissociation stimulators" -GDSs-). The first GDSs factors were identified in *Saccharomyces cerevisiae*: CDC25 and SDC25; CDC25 homologs were subsequently found in *Saccharomyces pombe* (Ste 6), *Drosophila melanogaster* (SON of seven-less gene product -SOS-) and in mammals (mCDC25 or GRF, mSOS, Vav). Proteins with the same function for the Rho subfamily of the Ras superfamily, as Dbl, and others with substrate specificity, as Smg, have also been dentified. The functional significance of such diversity of GDSs Ras is not yet clear. Nevertheless, and given the enormous Ras promiscuity in signal tranmission, such complexity could provide the necessary coordination of signal from different activated receptors by the tissue-specific way (63).

A third group of Ras regulatory proteins have recently been identified: GDP dissociation inhibitors (GDIs). Negative regulators of this type, functionally related, have been cloned and characterized in the Rab and Rho subfamilies, where they, besides, seem to play a role in the release of these proteins from the plasma membrane (28, 63).

4. Role of the Ras proteins in signal transduction

The activation of Ras proteins constitutes an essential and very well conserved step throughout the evolution in the transduction pathways of the mitogenic signals and of cellular differentiation mediated by tyrosine kinase receptors (RTKs) (fig. 4) (61, 63, 79, 86, 87). The action of a great variety of growth factors causes a rapid and transient increase of Ras active form level (12). This effect may be produced by inhibition of GAP activity or by stimulation of GDS activity, depending on the cell type and on the received stimulus (36, 88, 118). The p120GAP negative regulation could be brought directly, through its phosphorylation by RTKs ligand-activated, or indirectly via the phosphorylation of two p120GAP associated proteins, as p62 and p190, which would regulate the level of the GTPase stimulating activity of GAP. On the other hand, the positive regulation activity during stimulation could be brought by posttranslational modifications which would activate its catalytic function or through the regulation of its association with the plasma membrane (63). In this sense, a protein (Grb2 in mammals, Sem-5 in *C. elegans* and DrK in *D. melanogaster*) has been identified, which would act as an adaptor molecule between the activated RTKs and Ras (28). Thus, several studies carried out in mammals have shown that Grb2 would exist forming a cytoplasmatic complex with the mentioned SOS protein; the two of Grb2 SH3 domains would recognize proline rich SH3 binding motifs present in the C-termini of SOS. Upon ligand stimulation, the Grb2/SOS complex would associate with the autophosphorylated RTK via SH2 domain of Grb2, recruiting, thus, Grb2-associated SOS towards the plasma membrane, where it could stimulate Ras-GTP formation (10, 73). In addition to Grb2, a second protein, Shc, has been described, which by association with activated RTKs, would also increase the SOS concentration at the plasma membrane promoting activation of Ras proteins (fig. 4) (28, 63).

In spite of the fact that direct target of activated Ras is not known, numerous studies have shown the family of the serine/treonine kinase proteins activated by mitogens ("mitogen-activated protein kinases" -$MAPK_s$- or "extracellular-signal-regulated kinases" -Erks) as important downstream components of the Ras signal transduction pathway (63, 80). Ras activates the MAPKs through a cascade of serine/treonine kinases, the first of which is the product of the *raf-1* proto-oncogen, capable of associating directly with p21ras-GTP (3, 24, 70, 76). Raf-1 is also a potential substrate of the protein kinase C (PKC) (8), which can be activated in turn through Ras-GTP/GAP (6) or directly by diacilglycerol (DAG) and Ca^{++} (46, 52, 84). The experimental evidences suggest that the PKC could act as a conditional intermediary, according to the cellular systems, between Ras and Raf-1. Besides, in certain conditions, PKC can function to activate some of the Ras effects (48, 61, 69). After Raf-1 activation, the mechanism of which still presents some unknown factors, Raf-1 could function either as an activator of a MAPK kinase kinase (MAPKKK) or be itself the MAPKKK (76) to activate the MAPK kinases (or

MEKs) which would be the ones in charge of phosphorylating the MAPKs in threonines and tyrosines, activating them (1, 77). The MAPKs activation produces the phosphorylation and activation of factors of specific nuclear transcription factors (like c-Myc, c-Jun, TAL1 and p62TCF) which bind to specific places of the promotes region of determined genes stimulating their transcription. Likewise, the MAPKs activate other kinases that are critical for protein synthesis (like ribosomal S6 kinase -RSK- and the kinase -2 protein activated by MAPK- MAPKAP kinase-2) (7, 103). The MAPK pathway is not a single pathway and thus, three groups of $MAPK_s$ with different substrate specificities and regulated by different stimuli, have recently been established in mammals. Such subgroups are: The ERK subgroup, capable of phosphorylating and activating the transcription factor Elk-1, which binds to the serum response element; the JNK subgroup, which phosphorylates the N-terminal activation domain of the transcription factor c-Jun which binds to the AP-1 element; and the p38 subgroup, one of the functions of which is to phosphorylate small heat-shock proteins via a kinase cascade (25). The lineal schemes described of the intracellular signalling pathway which connects the tyrosine kinases, across Ras, with the MAPK kinase family cascade is only a simplification of the complex process that takes place at the physiological level. This complexity provides a mechanism of differential regulation of the mitogenic activation initiated by a wide spectrum of extracellular stimuli which are related to the specific phase of the cell cycle (11, 63, 76).

5. Ras oncogenes

The wide tissular distribution of the p21ras proteins and their fundamental role in the signal transduction pathways justify the fact that the *ras* transforming genes are the most frequently identified oncogenes in the development of human and animal tumors. The activation of any of the three *ras* genes has been detected with diverse frequencies in a great variety of human neoplasias: 70-95 % in adenocarcinomas of the exocrine pancreas, 65 % in colon carcinomas, 46 % in endometrial carcinomas, 30 % in lung carcinomas and 16 % in bladder carcinomas. In the case of mammary cancer, the incidence of activation of *ras* oncogenes is very low, having been related with worse prognosis (50, 91). It is important to note that the *ras* genes can acquire transforming properties not only by qualitative mechanisms but also by quantitative mechanisms associated to alterations of their expression (5, 91, 117). On the other hand, in spite of the fact that in determined cell lines *ras* oncogenes can confer metastasic properties (53), the metastasic phenotype is generally not considered to be an intrinsic property of such oncogenes (5).

As to the possible role of *ras* pseudogenes in carcinogenesis, it is worth noting that these pseudogenes possess multiple alterations with respect to the functional genes and that these could play a role in such process. The *c-Ha-ras2* pseudogene, for instance, presents, among the numerous base substitutions the same mutation in codon 12 as the retroviral *v-Ki-ras* oncogene. The fact that this residue is basic for the normal function of the protein and that it is found within a well conserved region, has led to hypothesize on the possible oncogenic potential of the pseudogene through a somatic recombination mechanism with other members of the *ras* family which could lead to the formation of an oncogenic hybrid gene (75, 96). Nevertheless, there are no proofs showing the possible *c-Ha-ras2* oncogenic potential.

5. 1. *ras* oncogenes in animal tumors

ras oncogenes have been activated in a reproducible way in a variey of experimental models of induced carcinogenesis in animals by chemical carcinogens. The altered sequences include, generally, codons 12, 13 and 59-61, typically by point mutations (5, 121).

In the mammary tumors induced in the Sprague-Dawley rat with dimethylbenz-(a)-anthracene (DMBA) (34, 35) the c-Ha-ras1 activation has been described as the result of a point mutation (A→T transversion) in the second base of the codon 61. This mutation generates a new *Xba*I restriction target in the locus, thereby it can be detected in Southern blot

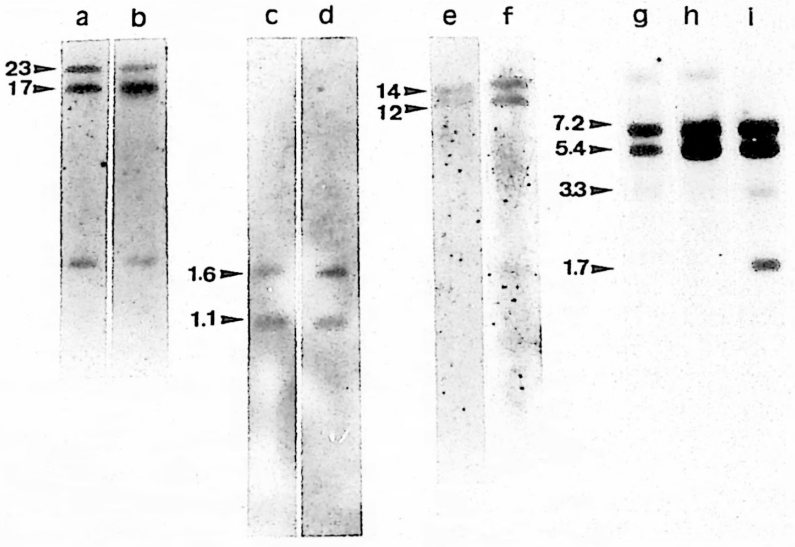

Fig. 5. Detection of c-Ha-ras1 and c-Ha-ras2 sequences in rat and of the point mutation induced by DMBA in c-Ha-ras1.

Southern blot of DNA from liver (lanes a, c, e, g) and DMBA-induced mammary adenocarcinomas (lanes b, d, f, h, i) from Sprague-Dawley rats hybridized to a viral *v-Ha-ras* probe. Methods: DNAs were purified by treatment with 0.3 mg/ml proteinase K and 0.1 mg/ml RNase and phenol-chloroform-isoamylalcohol extractions (25:24:1) (2). DNA samples (15 µg) were digested with restriction endonucleases *Eco*RI (lanes a, b), *Pst*I (lanes c, d), *Bam*HI (lanes e, f) and *Xba*I (lanes g, h, i), and analyzed by Southern blot assays (107). Digestion products were subjected to electrophoresis on 0.8 % agarose gels and were transferred by capillarity onto nylon positively-charged filterss. DNA was immobilized by incubation at +120 °C for 30 min and was hybridized to the same probe used in the RNA analysis (fig. 2) but labeled with digoxigenine (DIG-11-dUTP, Boehringer Manheim) by random priming, following the manufacturer's instructions. The hybridization, washes and immunological detection procedures were made as described by ENGLER-BLUM *et al.* (33). The hybridizations were basically in a solution containing 50 % deionized formamide/0.25 M Na_2HPO_4 pH 7.2/1 mM EDTA/ 0.5 % Blocking reagent/20 % SDS at +42 °C for 17 hours. Filters were washed 3 times for 20 minutes each at room temperature in a 20 mM Na_2HPO_4/1 mM EDTA/1 % SDS solution and for 1 hour in that same solution at +65 °C. During the detection the ENGLER-BLUM *et al.* conditions (33) were applied and the solutions recommended were used (buffer 1: O.1 M maleic acid/3 M NaCl, pH 8/0.3 % Tween-20; buffer 2: buffer 1/0.5 % Blocking reagent; buffer 3: 0.1 M Tris HCl pH 9.5/0.1 M NaCl/50 M $MgCl_2$). After treatment with the chemoluminescent PPD substrate, filters were incubated at +37 °C for 15 minutes and were exposed to autoradiography. The size of the bands is expressed in Kb.

hybridization analysis by a polymorphism in the length of this type of restriction fragments (RFLP *Xba*I). Figure 5 (lines g, h, i) shows the result of one of these analysis. The two additional bands of 3.3 and 1.7 Kb, which were detected in one of the two mammary tumors (lane i) analyzed for this RFLP, result from the appearance of a new *Xba*I target in the 5.4 Kb fragment which includes the *c-Ha-ras1* gene. These two bands are specific of the mutation as they were not observed either in normal tissue (line g) or in the non-carrier tumors (line h). The frequency with which this somatic mutation was detected was 30 %, a value similar to that described by other authors (65, 122, 128).

In mice, the same carcinogen (DMBA) induces skin tumors with identical mutation in 90 % of cases (90).

The induction of mammary adenocarcinomas in rats by a single dosis of nitroso-methylurea (NMU) during puberty provokes *Ha-ras1* activation in 86 % of the tumors. This activation is produced by mutation in the second nucleotide of the 12th codon (transition G →A) (94, 108).

On the other hand, the *c-Ki-ras2* activation, by point insertional mutations have been observed in 40 % of the renal mesenchymal tumors induced in the rats by a single dose of methyl(methoxymetyl)nitrosamine (DMN-OMe) (94, 108). The highest rates of activation of this oncogene (95 %) have been found in a pancreatic carcinogenesis model induced by N-nitrosobis(2-oxopropyl)amine (BOP) in Syrian hamsters. The *c-Ki-ras2* activation is also produced in this case by point mutation in the codons 12 or 13. In contrast, BOP-induced pancreas tumors in rats do not present the activated oncogen (119). Finally, the N-ras activation has also been observed next to the c-Ki-ras2 one in 60 % of the thymic lymphomas induced in mice by exposure to X rays or repeated treatments with NMU (42, 43). In this same experimental model with NMU, the loss of a normal *N-ras* allele in a tumor with an allele activated by C→A transversion in the first nucleotide of the 61 codon (44).

In general, it is not clear in what moment of the multistep process of the experimental carcinogenesis activation of *ras* oncogenes is produced. In some models it seems to appear during the early stages of tumor development (110) while in others, the low mutation frequency suggests that it could have to do with a later event (119). At any case, what is clear is that this activation would not be sufficient to trigger tumorigenesis. The cooperation with other genetic events, with normal physiological factors that specifically control development, and/or with environmental factors would be necessary (95, 106, 110).

5.2 ras oncogenes in human cancer

In spontaneous human tumors, *ras* activation frequency by point mutations in codons 12 or 61 is low (21), especially in the case of *c-Ha-ras1* (100). Thus, only in human bladder carcinoma cell lines could this mutated locus be detected, being a G→T transversion in the second nucleotide of codon 12 the responsible one for its transforming capacity (13, 92). In contrast *Ki-ras2* oncogene activated by this mutation, have been identified in several human neoplasias (colon, lung and pancreas) (14, 78, 105). An activated *N-ras* form has likewise been detected in human cell lines derived from sarcomas, neuroblastomas and leukemias (47).

It is very likely that expression of abnormally high levels of normal *ras* products may contribute to malignity, altering, for instance, the basic regulatory control mechanisms of cellular prolifera-

tion. Thus, *ras* gene expression in tumors has been shown to be often increased relative to normal tissues. Specifically, quantitative and immunological analyses indicate that the levels of *ras* transcripts or of p21 proteins in nearly 50 % of the human tumors are from 2 to 10 times higher than those of normal tissues. Surprisingly, low levels of p21ras have been found in metastasic tissues derived from primary colon carcinomas (5). In as much as to studies based on immunohistochemical techniques, the results follow the same course in spite of the high percentage of non-specificity. Nevertheless, the biological meaning of results of such studies is not clear. In general, increased expression of normal *ras* gene products has become involved in the development of certain animal tumors (124) and transformation of cells in culture (19, 39, 89). Nevertheless, in these cases the increase in the levels of both mRNA and the p21ras protein is in the order of 30 to 100 times higher than normal and it is associated, usually to the activity of a powerful viral promoter. On the other hand, as it was mentioned in section 3, increases in the level of *ras* transcripts of up to eight times may be associated to normal proliferative processes such as the hepatic regeneration in rat (41). The increase in the level of *ras* expression could, therefore, be the reflection of the high mitotic activity of the tumoral cells. Consequently, and up to the present, there exist no sufficient experimental bases to involve the moderate variation of the *ras* proto-oncogene expression in the neoplastic development of human tumours (5). Finally, there also exist controverted results about the specific relative weight of the qualitative alterations by mutation of specific codons and those of quantitative type. Thus, there exist data obtained in experiments carrried out *in vitro* that show that the increase in normal *ras* expression is insufficient *per se* for the complete cellular transformation and that mutations that induce changes in the p21 function/activity are necessary (93, 112). Nevertheless, other authors obtain, also *in vitro*, morphological and tumorigenic transformation with high normal p21 levels, although with lesser efficiency than with the mutated protein (89). In some cases it has even been suggested that point mutations are insufficient for the transforming function and that such activity depends especially on promoters or heterologous enhancers acquired by illegitimate recombination with other cellular or viral genes (15, 18).

The *ras* overexpression may be produced as a consequence of the alteration of their regulatory sequences or by gene amplification. In the first case, the alteration could be caused by proviral insertion or by point mutations. In human tumors, there exist no evidences for transcriptional activation of *ras* proto-oncogenes by insertional mutations. Contrariwise, the increase in the level of *c-Ha-ras1* mRNA by proviral insertion in an avian nephroblastoma induced by a myeloblastosis-associated virus, has been described (124). An increased *c-Ki-ras2* expression has also been found, associated with Friend virus integration in a bone marrow-derived mouse cell line (39). As to point mutations in regulatory regions, *c-Ha-ras1* overexpression in human primary mammary carcinomas has been detected in the absence of genetic reorganizations or amplification and point mutations, suggesting that such increased expression may be due to point mutations in cellular genes which regulate ras expression or in regulatory elements close to this locus (114). On the other hand, in T24/EJ human bladder carcinoma cells the *c-Ha-ras1* transforming allele in addition to the classic point activating mutation in the 12th codon (13, 29, 85, 99), it

contains a second alteration consisting in the A→G mutation in the nucleotide 2719. This mutation in the last intron results in a level of p21 protein ten times higher than normal and increase its transforming activity (16). This mutation and other ones seem to act abolishing the alternative splicing responsible for the generation of the non-transforming p19ras protein, and that operates to suppress the p21ras expression (17).

Significant amplification (≥ 10 fold) of *ras* genes associated with the overexpression of the protooncogen product has been observed in various human tumors. In general, the overall incidence of such genetic alteration in the human neoplasia does not seem to be higher than 1 % (5, 9, 38, 127). Among all the members of the *ras* family, *c-Ha-ras1* is the one that shows the best preserved number of copies. Some authors have proposed that the low frequency of *ras* gene amplification in tumors could be related to the greater transforming effect of low levels of the mutated protein with respect to the multiple copies of the normal protooncogene (89).

Finally, loss of *ras* heterozygosity has been described as a possible mechanism involved in the cell transformation and the tumorigenesis (97). Loss of one normal allele in cells carrying *ras* oncogenes has been detected with relative frequency in tumor cell lines, but only rarely in tumoral biopsies (44, 100). It is interesting to note loss of normal alleles in tumors in which the *ras* genes are not activated (49, 64, 127). Specifically, the loss of a *c-Ha-ras1* allele has been detected in up to 29 % of the studied cases, according to the type of tumor, and its frequency has been associated with more aggressive forms of primary carcinomas and with the development of distal metastases (127). In general, loss of *c-Ha-ras1* heterozygosity could not be correlated to genic expression changes (114, 127).

An interesting subject, is the possible relationship between *ras* genes and the individual predisposition to cancer. Such association was originally posed by KRONTIRIS *et al.* (64) in a population study on the frequency of the distinct *c-Ha-ras1* alleles in normal individuals versus cancer patients. Just as it was indicated when describing the *c-Ha-ras1* structure (see Section 1, fig. 1) the human gene possesses a region of repeated sequences in tandem -VTRs- at about 1.5 Kb from the 3'-terminus of its coding sequences. The variability in this region generated by the different number of repeated units produces different allele types which differ one from another by their size. This polymorphism can be detected by various RFLPs (restriction fragment length polymorphisms) (13, 49). In this way, a total of 20 different *c-Ha-ras1* alleles were identifies in that population study. Only 4 out of all of them were often found in the human population, both in healthy individuals and in cancer patients, for which reason they were classified as common alleles. The remaining alleles were occasionally detected and their frequency was greater in cancer patients than among the normal population. These allelic variants were inherited in a Mendelian manner and, therefore, did not seem to be a consequence of somatic events associated to tumoral development. As a whole, such results indicated that the rare *c-Ha-ras1* alleles could predispose the carrier individual to cancer (64). This hypothesis, however, has not been confirmed by other groups in various types of neoplasias (49).

The molecular bases of the greater susceptibility to cancer of rare alleles have been speculated upon. Thus, for example, the unusual VTRs could be inherited associated with determined mutations in *c-*

Ha-ras1, that could confer to it weakly oncogenic properties (64). In this sense, an association between an RFLP *Bam*HI generated by the VTR region and other RFLP *Pst*I based on the variable number of 6pb sequence repeats located in the first *c-Ha-ras1* intron, has recently been established (see fig. 1) (56). Another possible mechanism could come from the weak enhancer activity which the VTR region possesses. In this way, this region could alter the *c-Ha-ras1* expression or other neighboring loci predisposing certain cells to neoplasic growth (64). At present, the involvement of this allelic variability in the pathogenesis of human cancer is still to be determined.

The central role that *ras* plays in the cellular growth and its activation in at least 10 % of human tumors, turn the inhibition of the *ras* activity into a potential target for the therapeutic intervention in tumors whose growth is related to this oncogen. Nevertheless the abundant experimental evidences on the *ras* oncogen role on the cellular proliferation could be insufficient to attribute to it a causal role in the pathogenesis of the human cancer. In some cases, the *ras* oncogenes could be generated as a consequence of tumoral development. Furthermore it must not be forgotten that carcinogenesis is a multifactorial process and that it develops in multiple stages and that, therefore, *ras* activation could constitute just a link in the chain of events which lead to neoplastic transformation.

In spite of all that, the better knowledge of molecular and biochemical mechanisms, by which the *ras* oncogenes alter the normal proliferative cell programs, is essential to be able to establish possible connections between the activity of these oncogenes and the etiology and/or prognosis of the human cancer. That knowledge ought to allow, therefore, a better approach to cancer therapy in the future.

M. SOLANAS y E. ESCRICH. Ha-ras *en tejidos normales y tumorales: estructura, función y regulación.* J. Physiol. Biochem. (Rev. esp. Fisiol.), 52 (3), 173-192, 1996.

El gen *c-Ha-ras1* pertenece a una familia de genes eucariótica ubícua que codifica importantes moléculas implicadas en la transducción de señales mitogénicas y de diferenciación celular. La estructura de *c-Ha-ras1*, en cuatro exones codificantes y un exon 5' no codificante, está altamente conservada a lo largo de la evolución. Este gen, que genera un transcrito de 1,4 Kb, se expresa en prácticamente todos los linajes celulares de forma específica de tejido. La proteína codificada, de 189 aminoácidos y 21 KDa, es una pequeña proteína de unión a nucleótidos de guanina (GTP/ GDP), está asociada a la membrana plasmática y posee una baja actividad GTPasa intrínseca. p21ras funciona como un interruptor molecular que es activo unido a GTP e inactivo en su forma unida a GDP. Su actividad está muy estrechamente controlada en la célula por diversos mecanismos de control positivos y negativos. La familia de genes *ras* es la más frecuentemente implicada en el desarrollo de tumores humanos y animales. Se describen mecanismos de activación oncogénica cualitativos (mutaciones puntuales) y cuantitativos (sobreexpresión). Asímismo, se sugiere la posible relación entre determinados alelos *Ha-ras1* humanos y la predisposición al cáncer.

Palabras clave: *Ha-ras*, p21ras, Oncogen.

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