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21. I thank J. P. Eubanks for technical support, D. J. Weaver for secretarial support, and H. W. Dunn for x-ray fluorescence analysis. I also thank O. S. Andersen, W. Arnold, J. Braunstein, G. M. Brown, B. Z. Egan, A. I. Krasna, M. E. Reeves, and J. Woodward for comments and criticism. Supported by the Office of Basic Energy Sciences, Department of Energy, under contract DE-ACO5-840R21400 with Martin Marietta Energy Systems, Inc.

8 July 1985; revised 26 September 1985

A New Class of Steroids Inhibits Angiogenesis in the Presence of Heparin or a Heparin Fragment

Abstract. *Steroids that lack glucocorticoid or mineralocorticoid activity were found to inhibit angiogenesis in the presence of heparin or specific heparin fragments. This newly discovered steroid function appears to be governed by distinct structural configurations of the pregnane nucleus. These compounds are here named angiostatic steroids.*

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In an earlier study, we found that cortisone or hydrocortisone when administered with heparin or a heparin fragment inhibited the growth of new capillary blood vessels in the chick embryo, in the rabbit cornea, and in some mouse tumors (*1*). Dexamethasone appeared to have little or no anti-angiogenic activity in the presence of exogenous heparin, even though its glucocorticoid activity is about 30 times that of hydrocortisone. This apparent paradox suggested that the heparin-dependent anti-angiogenic function of these corticoids (corticosteroids) could be independent of their glucocorticoid function. We subsequently tested this hypothesis by substituting 11α-hydrocortisone (epicortisol) for hydrocortisone. Epicortisol is the biologically inactive stereoisomer of hydrocortisone (*2*). The α-position of the 11-hydroxyl group eliminates glucocorticoid and mineralocorticoid activity. Epicortisol retained anti-angiogenic activity in the presence of heparin when tested in the chick embryo (*3*).

We now show that there is a minimum essential structure for a new class of steroids which inhibit angiogenesis in the presence of heparin or a fragment of heparin that has no anticoagulant activity. Within this classification are steroids for which heparin-dependent anti-angiogenesis is the only recognized biological function. This new steroid function appears to be independent of glucocorticoid and mineralocorticoid activity.

All compounds were tested for their capacity to inhibit angiogenesis in the chorioallantoic membrane of the 6-day shell-less chick embryo. Each steroid, with and without heparin, was dissolved or suspended in 10 μl of methylcellulose (0.45 percent) which was then air-dried to a disk of 2 mm in diameter and implanted on the chorioallantoic membrane (Fig. 1). An optimum heparin concentration (Hepar, Inc.) was found to be 50 μg per 10 μl in the presence of hydrocortisone-21-phosphate (Sigma) (60 μg per 10 μl) (*4*). This combination was used as a positive control. A dose-dilution curve (1 to 200 μg) was generated for each test steroid in the presence of heparin. Each steroid was also tested alone at 200 μg, and alone at the concentration that had been most effective in the presence of heparin. The end point for angiogenesis inhibition was an avascular zone of 4 mm in diameter or greater when the embryo was examined 48 hours after implantation of the test compound (Fig. 2). At least 20 embryos were examined for each concentration of a steroid. Thus, 30 to 35 embryos received implants for each concentration, and 180 to 210 embryos were used for each steroid, not including the embryos used for the hydrocortisone-heparin controls. Throughout the year required for this study, the maximum anti-angiogenic activity of the hydrocortisone-heparin controls varied no more than 5 percent from week to week. Anti-angiogenic activity for each steroid was determined from its dose-response curve (Fig. 3).

At its optimum concentration, hydrocortisone (with heparin) produced avascular zones in 49 to 57 percent of the embryos. Higher concentrations (70 to 200 μg) resulted in reduced inhibition of angiogenesis and the appearance of an angiogenic reaction on the chorioallantoic membrane. This mild angiogenic reaction at high concentrations was increased in the absence of heparin but was not observed with steroids that lacked glucocorticoid activity. Neither heparin alone nor any steroid alone inhibited angiogenesis, with the exception of epicortisol, which at its highest dose (200 μg) displayed less than one-tenth of the anti-angiogenic activity that it produced with heparin.

The anti-angiogenic activity of epicortisol in the presence of heparin increased in a concentration-dependent pattern up to 200 μg. Maximum anti-angiogenic activity was about 28 percent that of hydrocortisone, while glucocorticoid and mineralocorticoid activity were completely lacking (Fig. 3). When the 11-hydroxyl group was absent, as in cortexolone, peak anti-angiogenic activity was retained at about 24 percent of hydrocortisone activity (Fig. 3). The glucocorticoid and mineralocorticoid activities of cortexolone are negligible. When both the 11-hydroxyl group and the 21-hydroxyl group were absent, as in 17α-hydroxyprogesterone, peak anti-angiogenic activity remained approximately equivalent to that of hydrocortisone (Fig. 3). This compound has essentially no glucocorticoid or mineralocorticoid activity. However, when the 17-hydroxyl group was absent, as in corticosterone, maximum anti-angiogenic activity

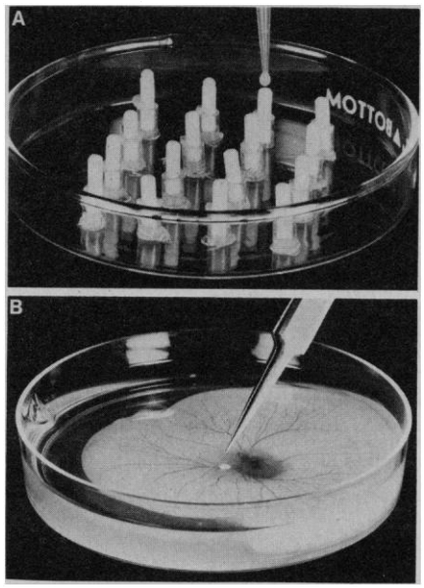

Fig. 1. (A) A 0.45 to 0.50 percent (weight to volume) solution of methylcellulose (4000 centipoise) in distilled water is autoclaved for 30 minutes and stirred at 4°C for 48 hours. Test substances are dissolved to a final concentration in a 10-μl aliquot, or (if insoluble), made into a fine suspension by shaking overnight in a nylon tube (Nunc) with glass beads. Aliquots of 10 μl are pipetted onto Teflon rods (outer diameter, 3.2 mm; length, 1.2 cm) held upright in glass tubing (inner diameter, 3.25 mm; outer diameter, 5 mm; length, 6 mm) cemented to glass petri dishes with Silastic adhesive. After being dried for 3 to 5 hours in a sterile hood, the disks, 2.2 ± 0.24 mm in diameter, are used immediately or within 2 to 3 days. (B) Six-day shell-less chick embryo at 37°C in 3 percent CO_2 humidified incubator prepared by modification of our previous method (*9*). On day 3 embryos are removed from their shell to petri dishes (Falcon 1005) under a sterile hood. Two hundred embryos can be prepared in 3 hours. Methylcellulose disks are implanted on the 5½- or 6-day chorioallantoic membrane when its diameter is 8 to 12 mm. The disk is placed on the outer third of the membrane where capillaries are still growing; that is, where widely spaced hexagonal capillaries appear instead of dense capillary networks. Best results are obtained when implantation is carried out in a clean room with 50 to 55 percent humidity at 27° to 32°C, with a stereoscope at ×7 to ×10. In an average week, 87 percent of the embryos survive removal from their shell; 71 percent are alive for implantation; and 68 percent are alive on day 8 for examination of avascular zones.

was reduced to about 24 percent of the activity of hydrocortisone. In desoxycorticosterone, where the 11- and 17-hydroxyl groups are both absent, anti-angiogenic activity was reduced to 17 percent of hydrocortisone activity (Fig. 3). Desoxycorticosterone is a mineralocorticoid that lacks glucocorticoid activity.

When the 11-, 17-, and 21-hydroxyl groups were absent, as in progesterone, anti-angiogenic activity was eliminated (Fig. 3). Anti-angiogenic activity was also virtually eliminated in estrone, in which carbons 20 and 21 are absent, although testosterone at high concentrations (100 to 200 μg per 10 μl), showed low levels of anti-angiogenic activity (Fig. 3). These compounds have neither glucocorticoid nor mineralocorticoid activity. Pregnenolone, a derivative of cholesterol and a precursor for the biosynthesis of the adrenal corticoids, did not inhibit angiogenesis at any concentration, with or without heparin. Pregnenolone has no glucocorticoid or mineralocorticoid activity (Fig. 3).

Steroids are mainly inactivated in the liver by the enzymatic reduction of the 4,5 double bond in the A ring to form the dihydrosteroid derivative (*5*). The dihydro derivative is converted to a tetrahydro derivative by the enzymatic reduction of the 3-oxo group to a 3-hydroxyl group. This compound is then conjugated with glucuronic acid to form a water-soluble product that is excreted by the kidney. The dihydro and tetrahydro derivatives of cortisone are considered to be biologically inactive (*5*). However, we found that both the dihydro (not shown) and the tetrahydro derivatives (Fig. 3), retained anti-angiogenic activity. In fact, the peak anti-angiogenic activity of the tetrahydro derivative (in the presence of heparin) was twice that of hydrocortisone.

Thus, we can now assign a function to a natural product of steroid metabolism that has been previously thought to be biologically inactive.

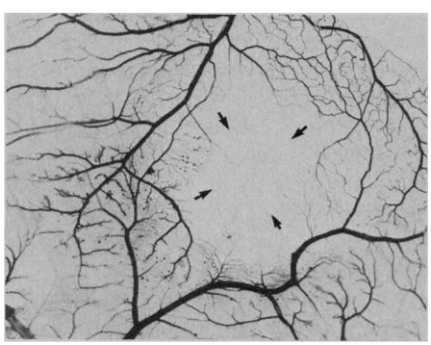

Fig. 2. Avascular zone in the 8-day chorioallantoic membrane, 48 hours after implantation of methylcellulose disk containing epicortisol (200 μg) and heparin (Hepar; 50 μg). Embryos are observed with a stereoscope at ×10 to ×15 for the presence or absence of capillaries around the disk. The maximum diameter of this zone is approximately 4.4 mm. By 60 hours after implantation, new capillaries usually begin to reenter this avascular zone. Embryos are graded at 48 hours as (−), normal capillaries beneath the disk; (±), area beneath disk is avascular (a zone of about 2 mm); (+), area of avascularity extends beyond the disk (diameter of up to 4 mm or more). India ink was injected.

The synthetic steroids exhibited greater anti-angiogenic activity than most of the natural steroids. For example, the anti-angiogenic activity of triamcinolone was about twice that of hydrocortisone. Triamcinolone has about 25 times the glucocorticoid activity of hydrocortisone. A synthetic steroid that has no glucocorticoid or mineralocorticoid activity, 6α-fluoro-17,21-dihydroxy-16β-methyl-pregna-4,9,(11)-diene-3,20-dione (Upjohn), had approximately nine times the anti-angiogenic activity of hydrocortisone (in the presence of heparin). However, when dexamethasone was originally tested at the concentrations that were maximally effective for hydrocortisone (50 to 60 μg), no anti-angiogenic activity was observed (*1*). In the present study, after dose-dilution curves had been completed for the natural steroids, we noticed that the highest anti-angiogenic activity for a given steroid often occurred at a lower concentration than the highest concentration tested. Therefore, we reexamined dexamethasone at concentrations down to 0.05 μg per 10 μl. At 2 μg there was a sharp peak of anti-angiogenic activity, which was 24 times that of hydrocortisone. However, from 3 to 200 μg, dexamethasone showed no anti-angiogenic activity. Concentrations above 100 μg were toxic, and 200 μg killed all embryos.

Anti-angiogenic steroids lacking glucocorticoid and mineralocorticoid activity have now been tested against neovascularization induced by V2 carcinoma in the rabbit cornea and their activity was found to be equivalent to that of the glucocorticoids previously reported (*1*). For example, when the steroid 6α-fluoro-17,21-dihydroxy-16β-methyl-pregna-4,9,(11)-diene-3,20-dione was tested with a hexasaccharide-rich heparin-fragment preparation [obtained by nitrous acid cleavage of heparin (Choay)], the maximum capillary growth rate at 14 days was: hexasaccharide alone, 0.14 mm/day; empty polymer, 0.10 mm/day; steroid alone, 0.04 mm/day; hexasaccharide plus steroid, 0.01 mm/day ($P < 0.05$). These compounds have not yet been tested by systemic administration in tumor-bearing mice.

Taken together, these experiments show that heparin-dependent anti-angiogenic activity does not correlate with the other known functions of the corticoids and appears to be independent of glucocorticoid and mineralocorticoid activity. These studies also demonstrate the existence of a class of steroids for which

inhibition of angiogenesis appears to be the principal function known so far. We suggest the name "angiostatic" steroids for these compounds. Other names (for example, angiotropic, or angiolytic) could be used. Included in this new class of steroids are the dihydro and tetrahydro metabolites of cortisone. Low levels of these steroids in the circulation may cooperate with heparin or heparin-like molecules normally found on the endothelial cell surface (*6*) to act as physiological inhibitors of endothelial cell turnover. If this idea is supported by additional evidence, the implication is that certain corticoid metabolites may also be growth regulatory molecules for vascular endothelial cells.

The available data reveal that anti-angiogenic activity is associated with the pregnane structure and governed mainly by structural components on the D ring. The 4,5 double bond in the A ring and the 11-hydroxyl on the C ring are not essential for anti-angiogenic activity. However, absence of the 17-hydroxyl and of carbons 20 and 21 on the D ring leads to successive reduction of anti-angiogenic activity.

It is possible to make synthetic steroids having only anti-angiogenic activity and in which this activity is significantly increased over that of the natural corticosteroids. Those steroids that have low-level anti-angiogenic activity in the absence of exogenous heparin may be synergized by local endogenous heparin or heparin-like molecules.

We previously reported that the contribution of heparin to the anti-angiogenic property of certain steroids was independent of its anticoagulant activity and that a non-anticoagulant hexasaccharide fragment produced by enzymatic cleavage of heparin showed optimum anti-angiogenic activity when administered with a corticoid (*1*). We have now found that a synthetic pentasaccharide fragment of heparin that had no effect against coagulation factors (Choay) (*7*), also inhibited angiogenesis with corticoids (Fig. 4). This counters the argument that the anti-angiogenic activity of heparin could be due to some nonheparin contaminant of commercial heparin. It also demonstrates that a synthetic heparin fragment and a synthetic steroid, each without recognizable biological activity, can cooperate to inhibit angiogenesis.

The mechanism by which angiostatic steroids inhibit capillary proliferation in the presence of heparin is unknown. However, Ingber *et al.* (*8*) observed that treatment of growing capillaries in the chorioallantoic membrane with heparin-steroid combinations resulted in dissolution of basement membrane only along regressing capillaries. Neither basement

	Anti-angiogenic	Gluco-	Mineralo-
Hydrocortisone	361	11	11
11α-Epihydro-cortisol	102	0	0
Cortexolone	86	0*	0*
17α-Hydroxy-progesterone	397	0*	0*
Corticosterone	86	0.3	1.6
Desoxycorticosterone	62	0	10
Progesterone	0	0	0
Testosterone	25	0	0
Estrone	13	0	0
Pregnenolone	0	0	0
Tetrahydro S	704	0	0

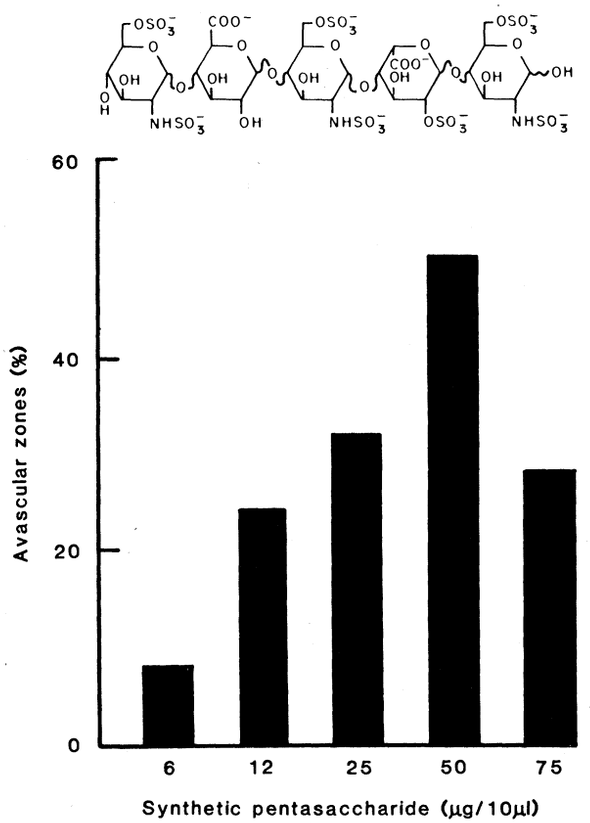

Fig. 3 (left). Comparison of anti-angiogenic index with glucocorticoid and mineralocorticoid activity. Steroids were tested at concentrations of 1, 5, 25, 50, 100, and 200 μg per 10 μl in the presence of heparin (Hepar; 50 μg/10 μl) with at least 20 embryos per concentration. The anti-angiogenic index was determined as the percentage of embryos with (+)-avascular zones at the most active dose, divided by the most active dose (expressed in micromoles). [Example: for hydrocortisone, the most active dose was 0.166 μmol (that is, 60 μg per 362.5 M_r; and the anti-angiogenic index was 60/0.166 = 361.] The index of glucocorticoid activity is based on deposition of glycogen in the liver; the index of mineralocorticoid activity is based on sodium excretion in the urine. These values are derived from reviews listed in (*5*). The asterisk indicates the glucocorticoid and mineralocorticoid activities of cortexolone and of 17α-hydroxyprogesterone are virtually absent. Fig. 4 (right). Dose-response curve of synthetic heparin pentasaccharide (Choay), which has no activity against coagulation factors, in the presence of hydrocortisone (50 μg/10 μl) implanted on the 6-day chorioallantoic membrane. Only avascular zones 4 mm in diameter or greater are presented. Neither pentasaccharide alone nor hydrocortisone alone produced avascular zones. The percentages of avascular zones observed at 75, 50, 25, and 12 μg of the pentasaccharide are significantly different from controls at $P < 0.001$ and the 6-μg dose of pentasaccharide at $P < 0.01$, as determined by the χ^2 test; 6 μg is significantly different from 50 μg at $P < 0.001$. Data are from three separate experiments.

membranes of large vessels nor neighboring ectodermal or endodermal basement membranes were affected. It is possible that loss of basement membrane scaffolding to which anchorage-dependent endothelial cells are normally attached may be involved in the associated capillary involution.

Our findings open the possibility of the chemical manipulation of angiogenesis. For example, this new class of steroids, when administered with an appropriate heparin fragment, may be of potential therapeutic benefit in some diseases dominated by a pathologic process which is angiogenesis-dependent.

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4. Anti-angiogenic activity of heparin (in the presence of steroids) varies with the manufacturer and the heparin lot. For example, at a concentration 25 μg/10 μl, the percentage of embryos with avascular zones was: Sigma 25 percent; Hepar 53 percent; Abbott Panheprin 89 percent (in the presence of hydrocortisone, 50 μg/10 μl); ($P < 0.05$ for three experiments). Therefore, when used locally in the chick embryo or in the rabbit cornea, heparins from different sources can be made effective by simply increasing the concentration of a less active heparin. This is not always possible when heparin is administered orally to achieve systemic anti-angiogenesis. When orally administered heparin was used (with corticosteroids) against mouse tumors (1), very high doses of heparin increased tumor growth, presumably due to enhancement of angiogenesis. Only Panheprin (Abbott) and, for some tumors, heparin from Hepar, Inc. were anti-angiogenic at a sufficiently low dose to inhibit tumor growth. Panheprin is no longer being manufactured.
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10. We thank D. Ingber, W. Risau and B. Zetter for helpful discussions; N. Chen for advice on steroid structures and for model building; D. Lund for the corneal implantations; W. Wierenga (Upjohn, Kalamazoo, Mich.), for the steroids; J. Choay (Choay Institute, Paris) for the synthetic pentasaccharide and the chemically cleaved heparin hexasaccharide. We also thank F. Barker for suggesting the term angiostatic; G. Jackson for the skill and patience required to prepare more than 16,000 shell-less chick embryos; B. Sabga for help in preparation and implantation of the methylcellulose disks; and P. Breen for manuscript preparation. Supported by USPHS grant R01-CA37395 and by a grant to Harvard University from the Monsanto Co.

23 July 1985; accepted 21 October 1985

Promoter Region of the Human Harvey *ras* Proto-oncogene: Similarity to the EGF Receptor Proto-oncogene Promoter

Abstract. *Regulation of transcription of members of the* ras *gene family undoubtably plays an important role in controlling cellular growth. Examination of this level of regulation requires identification of the promoter regions of the* ras *proto-oncogenes. Four major transcriptional start sites were detected in the human Harvey* ras *1 proto-oncogene. The promoter region contains neither a TATA box nor a CAAT box in their characteristic upstream positions, has an extremely high G+C content (80 percent), and contains multiple GC boxes including seven CCGCCC repeats and three repeats of the inverted complement, GGGCGG. This region has strong promoter activity when placed upstream from the chloramphenicol acetyl transferase gene and transfected into monkey CV1 cells. In these ways the Harvey* ras *1 proto-oncogene promoter resembles the promoter of the gene encoding the epidermal growth factor (EGF) receptor. The similarity between the two proto-oncogene promoters may be relevant to the mechanism by which the expression of such "growth control" genes is regulated.*

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The *ras* genes were first characterized as the transforming genes of the Harvey and Kirsten murine sarcoma viruses (v-Ha-*ras* and v-Ki-*ras*, respectively) (*1*). The H-*ras*, K-*ras*, N-*ras*, and *rho* genes constitute a family of conserved mammalian genes that encode structurally related proteins of approximately 21,000 daltons (p21) (*1–3*). Mutant *ras* genes encoding altered proteins are found in many human and rodent tumor cells and are capable of the malignant transformation of NIH 3T3 cells, an established murine cell line (*2, 4–9*). The mammalian *ras* gene products are associated with the cytoplasmic face of the cell membrane (*10, 11*), bind guanosine triphosphate (*12*), and exhibit a low level of guanosine triphosphatase activity (*13*). Little is known about the regulation of the expression of the *ras* genes. This is partly because the promoters of the *ras* genes have not been definitively identified even though the sequence at the 5′ terminus of some of the *ras* genes is known (*14, 15*).

We recently identified the promoter region of the gene coding for the proto-oncogene of the human epidermal growth factor (EGF) receptor (*16*). The EGF receptor is thought to be the cellular homolog of the v-*erbB* oncogene (*17–20*). The promoter region of the human EGF receptor proto-oncogene lacks a characteristic TATA box and CAAT box. It contains the repeat sequence CCGCCC, which is also found in the 21–base pair (bp) repeats of the simian virus (SV40) early promoter. We speculated that these features may be relevant to the mechanism by which the expression of some "growth control" genes is regulated (*16*). To investigate this possibility, we have identified and characterized the promoter region of another such gene—the human c-Ha-*ras* 1 gene.

To define the 5′ end of the c-Ha-*ras* 1 messenger RNA (mRNA), we first used primer extension analysis. A ^{32}P-labeled 51-bp Pvu II–Alu I DNA fragment (Fig. 1, nucleotides 1735 to 1684), which encodes a portion of the amino terminus of the *ras* protein, was hybridized to poly(A)$^+$ RNA of either the human epidermoid carcinoma cell line A431 or the mouse NIH 3T3 fibroblast cell line transformed by pEJ6.6 DNA, which contains the activated c-Ha-*ras* 1 gene of the human EJ bladder carcinoma (*21*). The primer was extended with reverse transcriptase, and the size of the products was determined by denaturing gel electrophoresis and autoradiography. When A431 mRNA was used, the most abundant extended products had lengths of 135, 142, 147, and 159 nucleotides (Fig. 2, lane 1). The mRNA of pEJ6.6-transformed NIH 3T3 cells served as template to synthesize the same set of extended products (Fig. 2, lane 2). The DNA sequence of these extended primers was determined and compared with the sequence of the c-Ha-*ras* 1 genomic clone reported earlier (*14, 15*). The comparison of the two sequences showed that an intron is located between nucleotides 577 and 1616 in the genomic clone (Fig. 1). The sequence at the exon 1–intron 1 boundary (nucleotide 576) is CGGT, whereas the intron 1–exon 2 boundary (nucleotide 1616) is AGGT. The sequences of the minor species of extended primers, which are longer than the four major species (see Fig. 2), are different from the sequence of the c-Ha-*ras* 1 genomic clone. We conclude that the