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Trans-acting factors regulate the expression of CD44 splice variants

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Variant isoforms of the cell surface glycoprotein CD44 (CD44v) are expressed during development, in selected adult tissues and in certain metastatic tumor cells. CD44v differ from the standard isoform (CD44s) by up to ten additional exon sequences included by alternative splicing. By cell fusion experiments, we have obtained evidence for the existence of cell-type specific *trans*-acting factors recruiting CD44 variant exon sequences. Stable cell hybrids of CD44s and CD44v expressing cells indicated a dominant mechanism for variant-exon inclusion. In transient interspecies heterokaryons of human keratinocytes and rat fibroblasts, the ability of the keratinocytes to include all variant exon sequences in CD44 was conferred completely on the rat fibroblast nucleus. Fusions of cells with complex CD44 splice patterns do not permit interpretation of splice control by the relative abundance of a single *trans*-acting factor, but rather by (a) positively acting factor(s) recruiting variant exon sequences in the 3′ to 5′ direction and additional factors selecting individual exons. Since the pancreatic carcinoma cell line BSp73ASML (in contrast to the cervix carcinoma cell lines SiHa and ME180) could not transfer its specific splice pattern in cell fusions, we conclude that in some tumors, splicing is also controlled by mutation of *cis*-acting recognition sites.

Keywords: alternative splicing/CD44 variants/exon-specific PCR/heterokaryons/*trans*-acting splice factors

Introduction

Splicing of pre-mRNA is a necessary step in eukaryotic gene expression. It serves to remove intron sequences from pre-mRNA and is carried out by large multicomponent complexes called the spliceosomes, which consist of a set of small nuclear ribonucleoproteins recognizing shared intron sequences (for review, see Moore *et al.*, 1993; Rio, 1993). Some genes give rise to more than one distinct protein isoform by alternative pre-mRNA splicing. Alternative splicing patterns can be regulated in the course of embryonic development (reviewed in Rio, 1993) or in response to specific physiological conditions (Deans *et al.*, 1989; Arch *et al.*, 1992). To achieve such splice regulation, regulatory factors could, in principle, interact with the general splicing machinery to change its mode of action, i.e. exon sequence retention versus exon skipping, or interact with cognate regulatory sequences in certain pre-mRNAs to tag individual exon sequences specifically for retention or skipping. Pre-mRNA sequences recognized by regulatory splice factors have been described for several *Drosophila* and mammalian gene transcripts. In *Drosophila*, such regulatory factors are activated during development (Black, 1992; Gallego *et al.*, 1992; Lavigueur *et al.*, 1993; Rio, 1993, and references therein; Watakabe *et al.*, 1993). The existence of splicing regulators specifically interacting with distinct pre-mRNAs would also allow the inclusion of exon sequences in the mRNA of one gene and the skipping of exon sequences in the mRNA of another gene within the same cell, as occurs for CD44 and CD45 during lymphocyte activation (Deans *et al.*, 1989; Arch *et al.*, 1992; Screaton *et al.*, 1995).

We will concentrate here on the alternative splicing of CD44. The term CD44 refers to a family of cell surface glycoproteins which are thought to be involved in processes like extracellular matrix binding, hemopoiesis, lymphocyte homing, cell migration, embryonic development and tumor metastasis (for reviews, see Haynes *et al.*, 1991; Underhill, 1992; Günthert, 1993; Lesley *et al.*, 1993; Sherman *et al.*, 1994, 1996; Moll *et al.*, 1996). Different CD44 isoforms are generated by alternative usage of amino acid sequences encoded by 10 variant exons. The variation occurs in the extracellular portion of the molecule, leading to a considerable number of theoretically possible primary structures (Screaton *et al.*, 1992; Günthert, 1993; Tölg *et al.*, 1993). Several of these variants have indeed been detected (see review by Sherman *et al.*, 1996). In contrast to the isoform lacking these variant exon sequences (standard form, CD44s) which is expressed on a variety of tissues and cell types, the expression of CD44 variant isoforms is restricted to the basal cells of squamous and glandular epithelia and to keratinocytes (Heider *et al.*, 1993; Mackay *et al.*, 1994). Moreover, cells of certain tumors express large CD44 splice variants, and their expression appears to promote metastasis formation during tumor progression (reviewed in Lesley *et al.*, 1993; Sherman *et al.*, 1994, 1996). Interestingly, expression of some of the variant isoforms associated with metastasizing tumor cells is transiently induced in lymphocytes upon antigenic stimulation (Arch *et al.*, 1992; Koopman *et al.*, 1993; Screaton *et al.*, 1995).

Since inclusion of variant exon sequences in CD44 mRNA is confined to certain cell types, there must be tight cell type-specific regulation of alternative splicing of CD44. Stringent regulation could be mediated by the presence of positive or negative *trans*-acting factors. Negative regulators would prevent the inclusion of variant exon sequences and thus give rise to the expression of the standard form of CD44 in most tissues, and would be inactivated in cells of certain differentiation pathways or

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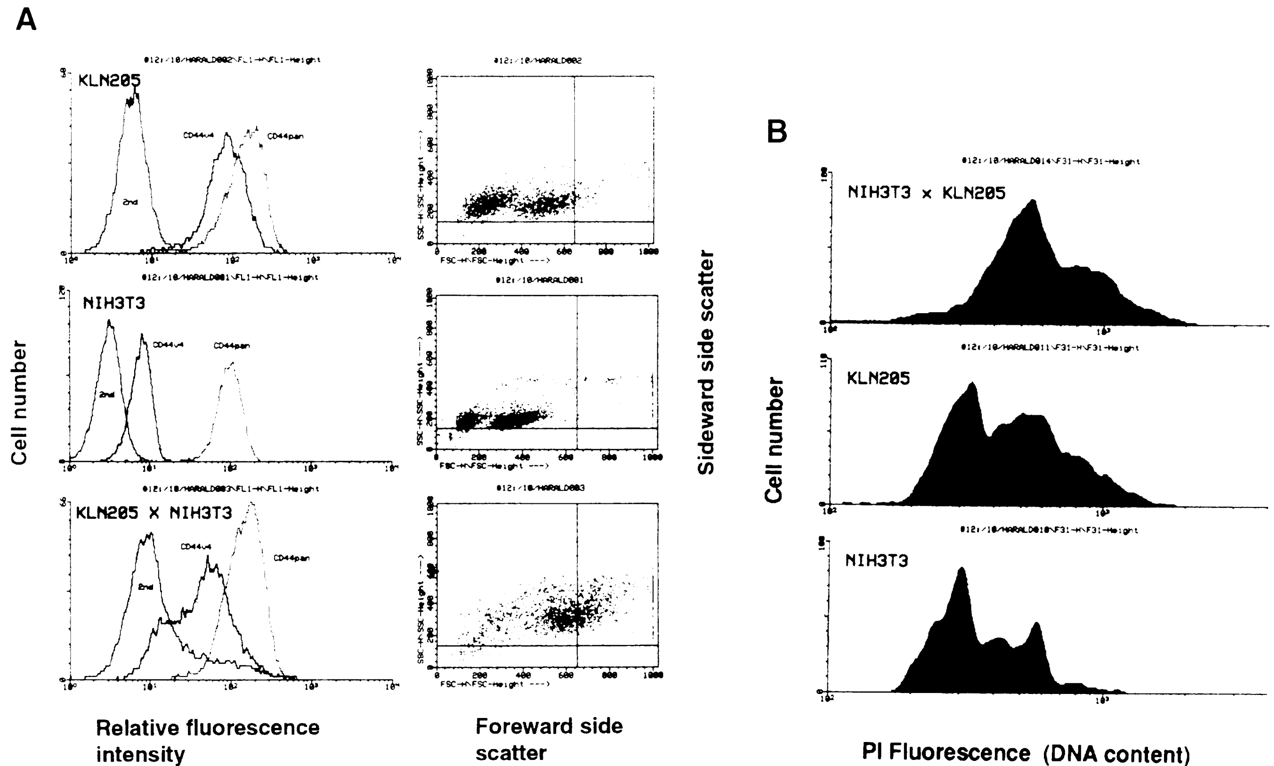

Fig. 1. Analysis of CD44 expression in stable cell hybrids between KLN205 mouse carcinoma cells and NIH3T3 mouse embryonal fibroblasts. (**A**) Left panel: flow cytometry analysis of CD44 expression on the parental KLN205 and NIH3T3 cells and in the KLN205×NIH3T3 cell hybrids. Cells were stained either with the second antibody (goat anti-rat coupled to FITC) alone (2nd) or with a rat anti-mouse CD44v4-specific antibody (mAb10D.1: CD44v4) and an antibody recognizing all CD44 isoforms (mAb IM7: CD44pan), respectively, followed by second antibody staining. Right panel: plot of the side scatter for the foreward scatter: the latter is a measure of cell size. (**B**) Flow cytometric measurement of the DNA content upon propidium iodide staining in the parental cells and the cell hybrids.

during tumor progression. Alternatively, choice of a specific splice isoform could be achieved by induced positive regulators that select the corresponding variant exon combination. In tumor cells, altered splicing patterns would then be caused by the up-regulation of such positive factors or by mutations in *cis*-acting elements recognized by the splicing regulators. The large variability of CD44 protein isoforms implies the existence of specific regulators capable of selecting certain variant exon combinations.

We report here on cell fusion experiments of partners expressing different CD44 splice patterns. The fusion experiments document the existence of dominant *trans*-acting factors acting on CD44 pre-mRNA. Such factors confer the ability to include variant exon sequences in CD44 mRNA and determine specific exon combinations.

Results

CD44 variant expression is a dominant feature

To examine whether the expression of CD44 variant isoforms is a dominant or a recessive characteristic of cells, we produced stable somatic cell hybrids (synkaryons). The CD44 expression pattern was then followed by both a monoclonal antibody (mAb) recognizing an N-terminal epitope present on all known CD44 molecules (IM7: panCD44) and thus also staining the abundant CD44s form, and a mAb directed against an epitope encoded by exon v4 (10D1, the first anti-mouse CD44v mAb we obtained; J.Sleeman, unpublished). NIH3T3 embryonal mouse fibroblasts expressing the standard form of CD44 (CD44s) plus a small amount of a variant molecule carrying a v4 exon epitope (see Figure 1A, left panel), were stably fused with KLN205 mouse carcinoma cells which express high levels of the CD44v4 epitope (for isoform nomenclature see Tölg *et al*., 1993) as determined by antibody staining using the anti-CD44v4-specific mAb 10D1 (see Figure 1A, left panel). To be able to select for stable cell hybrids, the parental cell lines had first been stably transfected with plasmids conferring resistance to G418 (NIH3T3) and puromycin (KLN205), respectively. Doubly resistant hybrid cells were selected. The parental cell lines and the cell hybrids were then analyzed for both total CD44 expression (staining with an mAb recognizing an N-terminal epitope: panCD44) and CD44v4 epitope expression by flow cytometry. By light scattering, the doubly resistant cells were significantly larger in size than the parental cells (Figure 1A, right panel) and their DNA content was roughly twice that of the parental cells (Figure 1B). This strongly suggests that the cells obtained by the double selection procedure were in fact cell hybrids. Both parental cell lines expressed CD44 as detected by staining with the CD44pan antibody (Figure 1A, left panel). As stated above, KLN205 cells carried high levels of an epitope encoded by exon v4, whereas the NIH3T3 cells were only slightly positive for this epitope (Figure 1A, left panel). The vast majority of the NIH3T3×KLN205 hybrid cells still showed high CD44v4 epitope expression similar to that of the parental KLN205 cells (Figure 1A, left panel), indicating that high CD44 variant expression is not suppressed. Thus, the result suggests that the

high expression phenotype of the KLN205 CD44 variant isoform is dominant over the NIH3T3 phenotype.

One could argue that a chromosome was lost in the hybrids. Although >100 doubly resistant clones were pooled and analyzed, making it unlikely that in each clone the same chromosome was lost, we cannot completely rule out that the loss of a chromosome encoding a putative repressor of variant exon inclusion, together with a possible growth advantage, could have resulted in CD44 variant-expressing cell hybrids.

Induction of variant CD44 isoforms in interspecies heterokaryons

To exclude the possibility of chromosomal loss and to distinguish whether there are *trans*-acting factors or *cis*-acting mutations mediating the inclusion of variant CD44 exon sequences, we generated transient interspecies heterokaryons. Heterokaryons were examined after cell fusion prior to cell division and prior to fusion of the nuclei (Blau *et al.*, 1983; Baron and Maniatis, 1986). Their nuclei are located in a common cytoplasm and, by using cells from different species in the fusion experiment and species-specific probes, it is possible to study the communication between the nuclei. Species differences can be exploited both by specific primers for PCR and by mAbs recognizing species-specific epitopes. If the notion of dominant variant expression obtained in the stable fusions were valid, dominant *trans*-acting factors should, also in the transient heterokaryons, confer the ability to include variant exon sequences in the CD44 mRNA to cells that normally skip these exons. In the example outlined in Figure 2A, we fused human immortalized keratinocytes (HaCaT), which produce high levels of CD44 isoforms containing variant exons v2–v10 (Hofmann *et al.*, 1991; Günthert, 1993; this study, see Figure 4A, left panel), with embryonal rat fibroblasts (Rat2) which synthesize CD44s and almost no CD44 variant isoforms (this study, see Figure 4A right panel). To be able to detect heterokaryons within the majority of unfused cells and of cell fusions of the same species, cells were selected that co-express both rat- and human-specific CD44 epitopes on the same cell surface (interspecies fusions; see Figure 2A). To this end, panCD44 antibodies specific for either rat (mAb Ox50) or human (mAb IM7) were bound and detected by secondary antibodies coupled to different fluorescent dyes. After 5 h of fusion by polyethylene glycol (PEG), typically ~3–4% of all cells carried both rat and human CD44 epitopes indicating cell fusion (labeled F in Figure 2B) whereas, when PEG was omitted, no CD44 doubly-positive cells were generated (data not shown). Using a biotinylated version of the anti-rat CD44v6-specific mAb 1.1ASML (Günthert *et al.*, 1991), detected by streptavidin coupled to a third fluorescent dye (see Figure 2A), the v6 epitope was barely detectable on the Rat2 cell population (labeled R in Figure 2C). The HaCaT population (labeled H in Figure 2C) also showed no staining, although HaCaT cells strongly express exon v6 as demonstrated by an anti-human CD44v6 antibody (data not shown), indicating the efficient discrimination between rat and human CD44v6 epitopes in the experiment. The heterokaryon population (labeled F in Figure 2C), however, showed clearly detectable staining with the rat-specific anti-v6 antibody. This induction of CD44 variants carrying the v6 epitope is not caused by an overall increase in the expression of rat CD44 and thus leaky splice control in these cells. In fact, overall CD44 levels determined by an antibody recognizing all rat CD44 isoforms (mAb Ox50) were consistently even somewhat lower on the heterokaryons than on the Rat2 cells (Figure 2D). Thus, the results indicate a change in splicing in the rat nucleus under the influence of factors from the other (human) nucleus.

Trans-acting factors determine the cell type-specific pattern of CD44 pre-mRNA splicing

The mAbs only indicate the inclusion of an exon, but do not define the exact exon composition of the variant CD44 isoforms. To explore whether the dominant factor(s) enforce a specific splice pattern, we analyzed the composition of mRNA. Rat and human CD44 RNA was distinguished by rat and human sequence-specific primers, respectively, complementary to the 5′ and 3′ constant portion of the CD44 cDNA. The human-specific primers did not yield RT–PCR products with RNA from Rat2 cells or Rat2 homotypic fusions (Figure 2E), but generated four distinct bands hybridizing to CD44 cDNA, with RNA from either HaCaT cells, HaCaT–Rat2 mixtures or HaCaT×Rat 2 heterokaryons prior to or after sorting by FACS. The largest of these bands also hybridized to a probe comprising exons v1–v3 (Figure 2E), consistent with the finding that HaCaT cells synthesize CD44v2–v10 RNA (Hofmann *et al.*, 1991). The analysis of PCR products generated with rat-specific primers clearly indicates the action of one or several *trans*-acting dominant factors. While RNA from Rat2, Rat2×Rat2 fusions and from HaCaT–Rat2 mixtures gave rise predominantly to an RT–PCR fragment of 1120 bp indicative of CD44s, the interspecies heterokaryons produced a larger band which also hybridized to the v1–v3 probe (Figure 2E). The size of this band is compatible with the presence of all variant exons. The factor(s) contributed by HaCaT thus seem to cause inclusion of all variant exon sequences in RNA derived from the Rat2 nucleus. CD44s mRNA is, however, still present in the heterokaryons, but a significant fraction of rat CD44 mRNA represented CD44v1–v10. We need to take into account that the PCR reaction with outside primers underrepresents the abundance of the larger RNA and that the sorting may not have resulted in complete lack of contamination by parental Rat2 cells.

To determine exactly the exon composition of the new rat transcript(s) induced by the splice regulator(s), we applied serial PCRs with specific primers for each of the variant exons (van Weering *et al.*, 1993). cDNA was synthesized by oligo(dT) priming and CD44 cDNAs were pre-amplified with either rat- or human-specific primers hybridizing to the 5′ and 3′ constant parts of CD44 cDNAs (see Figure 3). In subsequent PCRs, aliquots of the pre-amplified products were re-amplified between a primer of the 3′ constant region (C2A for rat, hs3′ for human cDNAs) and one of the variant exon-specific 5′ primers pv1–pv10, or a 5′ constant primer (C13, see Figure 3). Since exon v1 is not expressed in human cells (Screaton *et al.*, 1993) and exon v3 contains two alternative splice acceptor sites in human CD44 pre-RNA (Screaton *et al.*, 1992), human RNA was analyzed without primer v1 but with two exon v3-specific primers, pv3I and pv3II

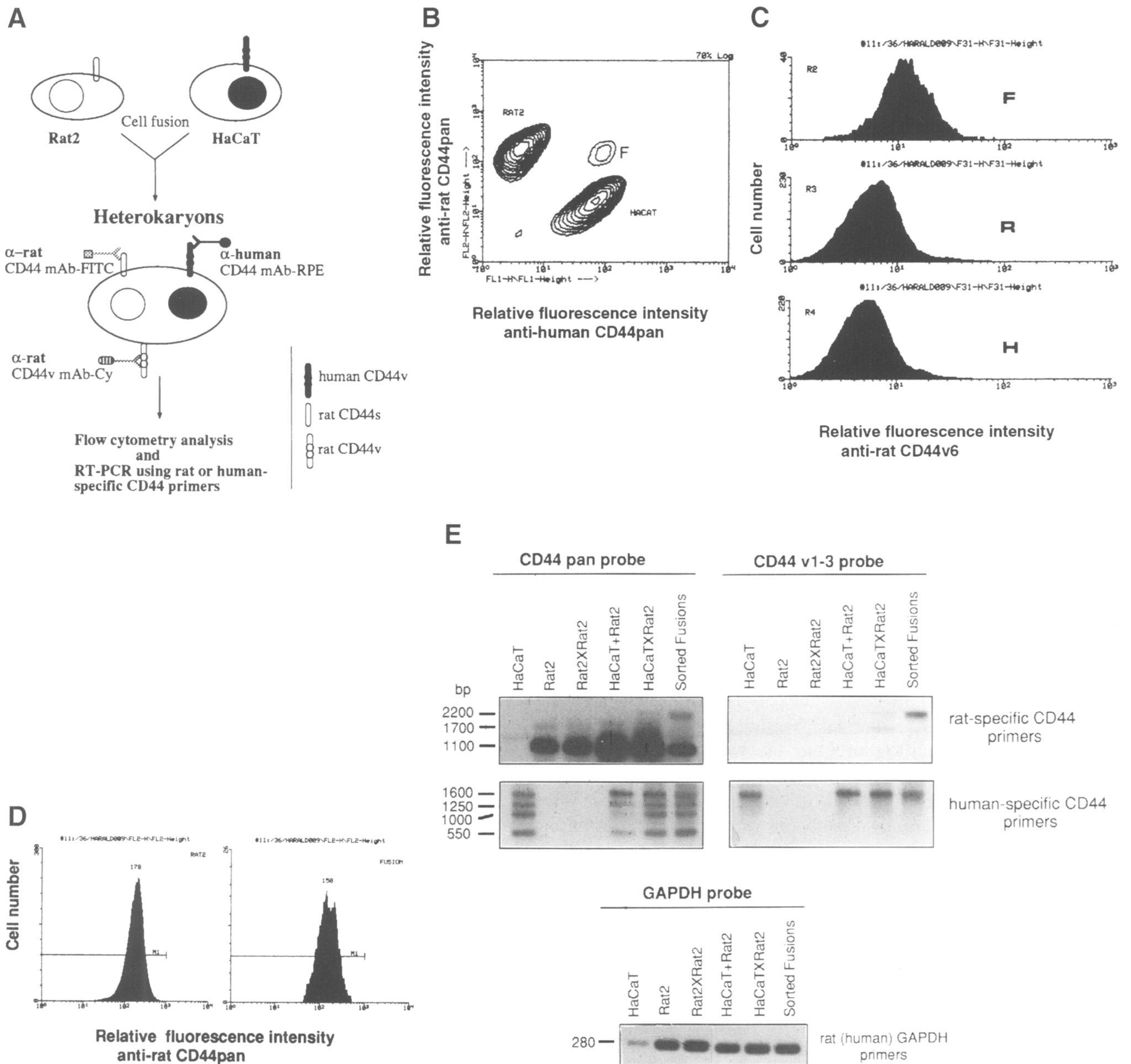

Fig. 2. Analysis of CD44 expression in transient heterokaryons of human HaCaT keratinocytes and Rat2 rat fibroblasts. (**A**) Schematic outline of the experiment. HaCaT and Rat2 cells were fused by PEG and the entire cell population was stained by a rat antibody (mAb IM7) recognizing human (but not rat) CD44 and by a mouse antibody specific for rat CD44 (mAb Ox50). Following staining with corresponding second antibodies coupled to fluorescein isothiocyanate (FITC) and R-phycoerythrin (RPE), respectively, rat CD44 variant (CD44v) expression was detected by a biotinylated mouse anti-rat CD44 antibody (mAb1.1ASML) recognizing an epitope encoded by variant exon 6 (v6) and streptavidin coupled to cychrome (Cy). In addition, expression of rat and human CD44 mRNA species was analyzed by RT–PCR [see (E) and Figure 4]. (**B**) Flow cytometry analysis of the cell populations in the cell fusion experiment. The parental HaCaT and Rat2 cell populations (containing also homo-fusions) and the HaCaT×Rat2 fusions (F) which express both human and rat CD44 are shown as a contour plot. (**C**) Flow cytometry analysis of rat CD44v6 expression (Cy fluorescence) on the HaCaT×Rat2 heterokaryon population (F), the Rat2 cell (R) and the HaCaT cell (H) populations shown in (B). (**D**) Flow cytometric analysis of overall rat CD44 expression levels in the heterokaryons (fusion) compared with the Rat2 cell population following staining with an antibody (mAb Ox50) recognizing all rat CD44 isoforms. Numbers over the peaks give the calculated means from the indicated interval (horizontal bars). (**E**) Semi-quantitative RT–PCR analysis of CD44 expression in HaCaT, Rat2 cells, Rat2 cells fused by PEG (Rat2×Rat2), HaCaT cells mixed and co-cultured (HaCaT+Rat2) or fused (HaCaT×Rat2) with Rat2 cells and FACS-sorted HaCaT×Rat2 heterokaryons (sorted fusions). cDNAs were amplified with either human-specific (hs5′ and hs3′) or rat-specific CD44 primers (rs5′ and rs3′, for primer positions see Figure 3), resolved by agarose gel electrophoresis, blotted and hybridized to the CD44 probes indicated. RT–PCR using GAPDH primers served as a control for the cDNA amounts used. Numbers on the left indicate fragment sizes in bp.

(van Weering *et al.*, 1993). The gels shown in Figures 4 and 5 were evaluated for two types of qualitative information. Lane C (lane 11) reveals the lengths of the amplification products between constant exon primers. As stated above, with increasing distance between the primers, reaction efficiency diminishes. Thus, the abundance of RNA for larger variants will be underestimated. The other type of information yields the variant exon composition (lanes 1–10) and, by the ladder-like increments by known exon sequence lengths, the exact order of variant exon

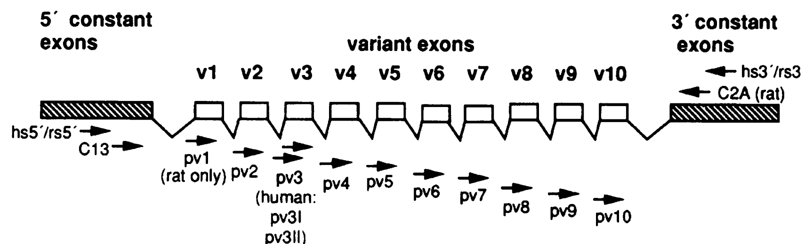

Fig. 3. Simplified scheme of the CD44 gene indicating the positions of the primers used in the exon-specific RT–PCR analysis. Hatched bars denote 5′ and 3′ constant exons (without introns), open bars represent the variant exons, the lines connecting the exons depict the intron sequences. Primer positions are indicated by arrowheads. hs5′ and hs3′, 5′ and 3′ human-specific primers used for pre-amplification; rs5′ and rs3′, rat-specific 5′ and 3′ primers used for pre-amplification; C13, pv1–pv10, 5′ primers used for exon-specific PCRs in combination with the 3′ primer C2A for analysis of rat cDNAs and hs3′ for human cDNAs, respectively. pv1 is not used in the analysis of human cDNAs since the exon v1 sequence is not expressed in human cells (Screaton *et al.*, 1993). Exon v3 is covered by two primers (v3I and v3II) due to the existence of an alternative splice acceptor site in the human v3 exon (van Weering *et al.*, 1993).

sequences in the RNA (note that sizes in lanes 1–10 do not correspond to those in lane 11 due to different 5′ primer positions).

Using human 5′ and 3′ constant region primers (C13 and hs3′; see Figure 3) for re-amplification, the parental HaCaT cells showed a weak 268 bp (calculated size) band corresponding to the standard CD44 mRNA, and several bands corresponding to variant CD44 mRNAs (Figure 4A, left panel, lane 11). Predominant variant products were 729, 925 and 1411 bp in size. The exon-specific PCR analysis with the primers v2–v10 indicates that all of the variant exons are expressed (lanes 1–10) and that all RNAs end with v10 as the last variant exon. Together with the size information of lane 11, we can conclude that HaCaT cells produce predominantly CD44v8–v10, CD44v6–v10 and CD44v2–v10. In contrast, re-amplification of parental Rat2 cDNA with the rat constant region primers (C13 and C2A, see Figure 3) resulted in one strong band (594 bp) corresponding to CD44s mRNA. Larger bands derived from variant CD44 mRNAs are barely detectable (Figure 4A, right panel, lane 11). PCR with the exon-specific primers pv1–pv10 likewise generated only very faint bands, suggesting that it is the standard form of CD44 which is expressed predominantly in the Rat2 cells. Very long exposures (not shown) revealed the presence of minute amounts of CD44v9,v10, CD44v8–v10 and CD44v6–v10 cDNAs.

RNA from the FACS-sorted HaCaT×Rat2 heterokaryons analyzed for rat transcripts using rat-specific CD44 primers, however, gave rise to a quite different result (Figure 4B, right panel). The constant region primers still amplify the major 594 bp standard product but, in addition, variant products are readily detectable (lane 11), the most prominent of which correspond in size to the rat variants CD44v6–v10 and CD44v1–v10. The exon-specific PCRs (lanes 1–10) indicate the presence of all variant exons and their continuous alignment up to CD44v1–v10 in the transcripts from the rat nucleus, a pattern quite similar to the one obtained using RNA from the parental (human) HaCaT cells (and human primers). Only the induced production of rat CD44v8–v10 is less pronounced than in the parental HaCaT cells. The pattern obtained was derived totally from rat CD44 mRNA since HaCaT RNA gave no signal at all using the rat primers (data not shown).

The results (summarized in Figure 4C) thus show that the keratinocyte-associated splicing pattern of CD44 mRNA can be conferred to fibroblasts by cell fusion, confirming that there are cell type-specific and positively acting factors regulating alternative CD44 mRNA splicing *in trans*. On the other hand, no changes could be detected in the splicing pattern of RNA from the HaCaT nucleus in the sorted heterokaryons by using human-specific CD44 primers for the analysis (Figure 4B, left panel), suggesting that the Rat2 fibroblast nucleus contains no negatively acting and dominant splice regulators capable of inhibiting the inclusion of variant CD44 exon sequences.

Exon-selective trans-acting factors?

In a listing of the frequency of occurrence of CD44 variant exons in various tissues, v10 appeared to be included in CD44 most frequently, and the occurrence of exons decreased in the 3′ to 5′ direction (Screaton *et al.*, 1993). One could therefore argue that the abundance of a single factor could determine progressive inclusion of exons operating from v10 to v1. The fusion experiments reported above could be interpreted to indicate the existence of such a factor. Its abundance in HaCaT cells would be so high that reduction to roughly one half by the cell fusion could not obliterate function, and all variant exon sequences were included in mature transcripts from both nuclei of the heterokaryons.

To explore whether, in addition, there are exon-specific factors, we fused cells with complex expression patterns. We generated transient heterokaryons with rat BSp73-ASML pancreatic carcinoma cells (Matzku *et al.*, 1983) which produce predominantly CD44v4–v7 and CD44v6–v7 (Rudy *et al.*, 1993; Figure 5A, left panel), minor amounts of CD44v2–v7 and CD44v8–v10, as well as minute quantities of several other splice variants, e.g. CD44v2–v10 and CD44v4–v8. In one series of fusions, we chose the human embryonic kidney cell line 293 which expresses CD44s, CD44v8–v10 and CD44v3. The 293 cells also appear to produce some CD44v6–v10 and CD44v6 (Figure 5A, right panel). Looking at the rat-specific RNA in sorted hybrid cells, it seems that the level of CD44v4–v10 is increased (Figure 5B, left panel). The 293-specific expression of single-exon variants CD44v3 and CD44v6 was not transferred. Also the CD44v4–v7 splice pattern typical of BSp73ASML cells was not extended in either direction by fusion with 293 cells. On the other hand, using human-specific primers, the heterokaryons show some qualitative differences from the original 293 splice pattern: CD44v6 disappears, and new

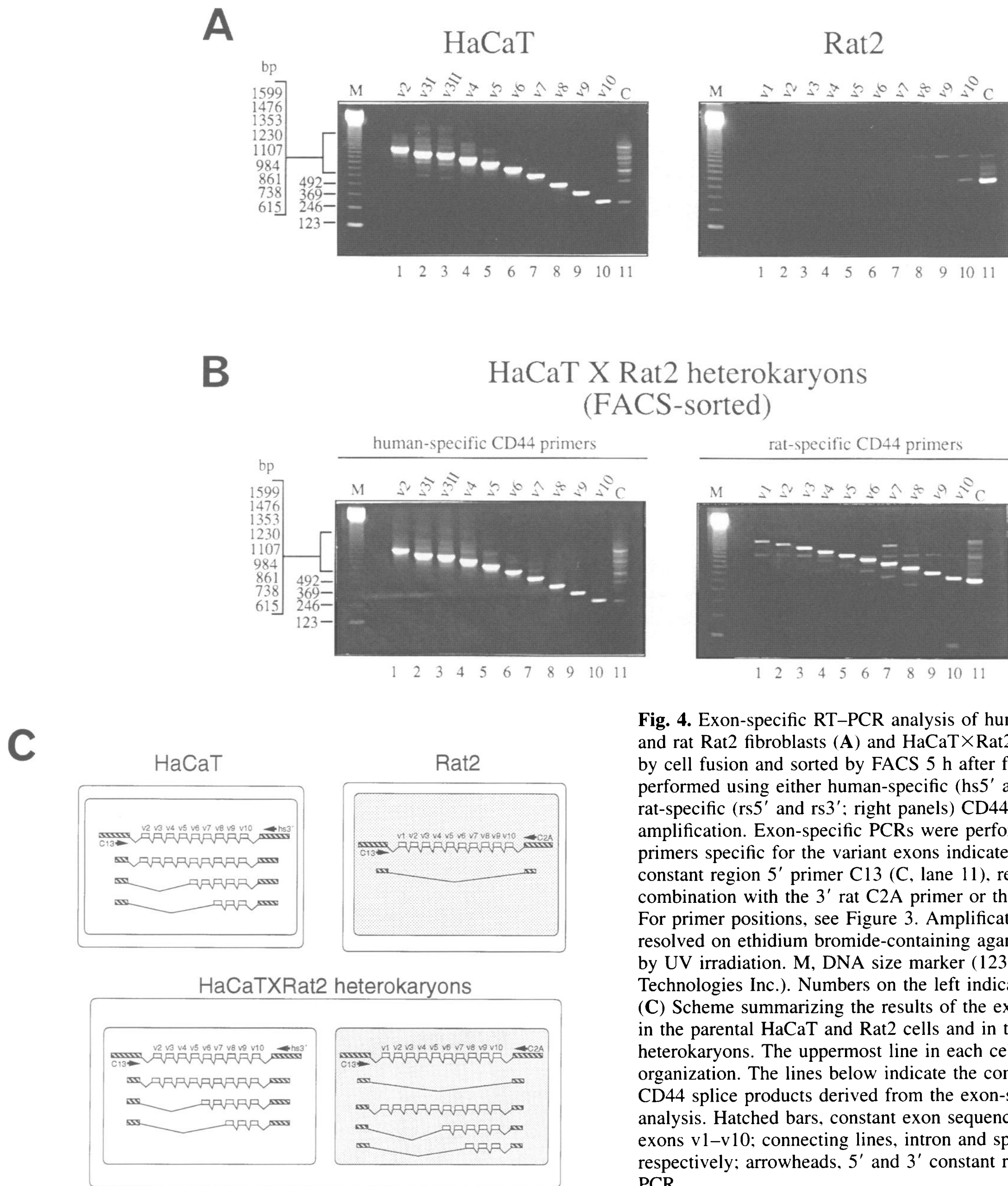

Fig. 4. Exon-specific RT–PCR analysis of human HaCaT keratinocytes and rat Rat2 fibroblasts (**A**) and HaCaT×Rat2 heterokaryons generated by cell fusion and sorted by FACS 5 h after fusion (**B**). PCRs were performed using either human-specific (hs5′ and hs3′; left panels) or rat-specific (rs5′ and rs3′; right panels) CD44 primers for pre-amplification. Exon-specific PCRs were performed using the 5′ primers specific for the variant exons indicated (lanes 1–10) and the constant region 5′ primer C13 (C, lane 11), respectively, in combination with the 3′ rat C2A primer or the 3′ human primer hs3′. For primer positions, see Figure 3. Amplification products were resolved on ethidium bromide-containing agarose gels and visualized by UV irradiation. M, DNA size marker (123 bp ladder, Life Technologies Inc.). Numbers on the left indicate marker sizes in bp. (**C**) Scheme summarizing the results of the exon-specific PCR analysis in the parental HaCaT and Rat2 cells and in the sorted HaCaT×Rat2 heterokaryons. The uppermost line in each cell shows the CD44 gene organization. The lines below indicate the composition of the major CD44 splice products derived from the exon-specific RT–PCR analysis. Hatched bars, constant exon sequences; open bars, variant exons v1–v10; connecting lines, intron and spliced intron sequences, respectively; arrowheads, 5′ and 3′ constant region primers used for PCR.

splice variants carrying CD44v8 and CD44v8–v9 occur. Most interestingly, however, a variant containing exons v4 and v5, which were not expressed at all in the parental 293 cells, was now detectable upon cell fusion (Figure 5B, right panel).

Although the interpretation of these complex expression patterns is rather difficult, we would like to point out that it is striking that the CD44v4–v7 splice of the BSp73ASML carcinoma cells is not transferred to the 293 cells, while the exon combination v4,v5, which is present in the CD44 mRNA of BSp73ASML cells, is newly induced in the 293 nucleus, but not joined to the large CD44v6–v10 RNA. This may imply that there are factors selecting distinct exons in addition to one including exons in the 3′ to 5′ direction. In BSp73ASML, a *cis*-acting mutation may prevent exon sequence linkage 3′ of v7 in one allele while the other seems to permit this joining.

In a second series of fusions, HaCaT cells were the partners of BSp73ASML. In part, the result confirmed those obtained with 293 cells. In the rat nucleus, there was no extension of the CD44v4–v7 splice in the 3′ direction, and the v4–v7 splice was not conferred on the HaCaT nucleus (Figure 5C, compare with A). Rat-specific RNAs from the sorted heterokaryons yielded a more diverse pattern of splice products (compare lane 11 of Figure 5C, left panel with lane 11 of Figure 5A). The analysis with human-specific primers revealed no change from the HaCaT pattern of CD44 expression (Figure 5C) as if in HaCaT the high level of a 3′ to 5′ splice regulator prevented the influence of a putative exon selector as postulated for the fusion with 293 cells.

Several other tumor cell lines were subjected to transient heterokaryon analysis using NIH3T3 cells as partners. We found evidence for *trans*-acting factors in the MTLn3 rat

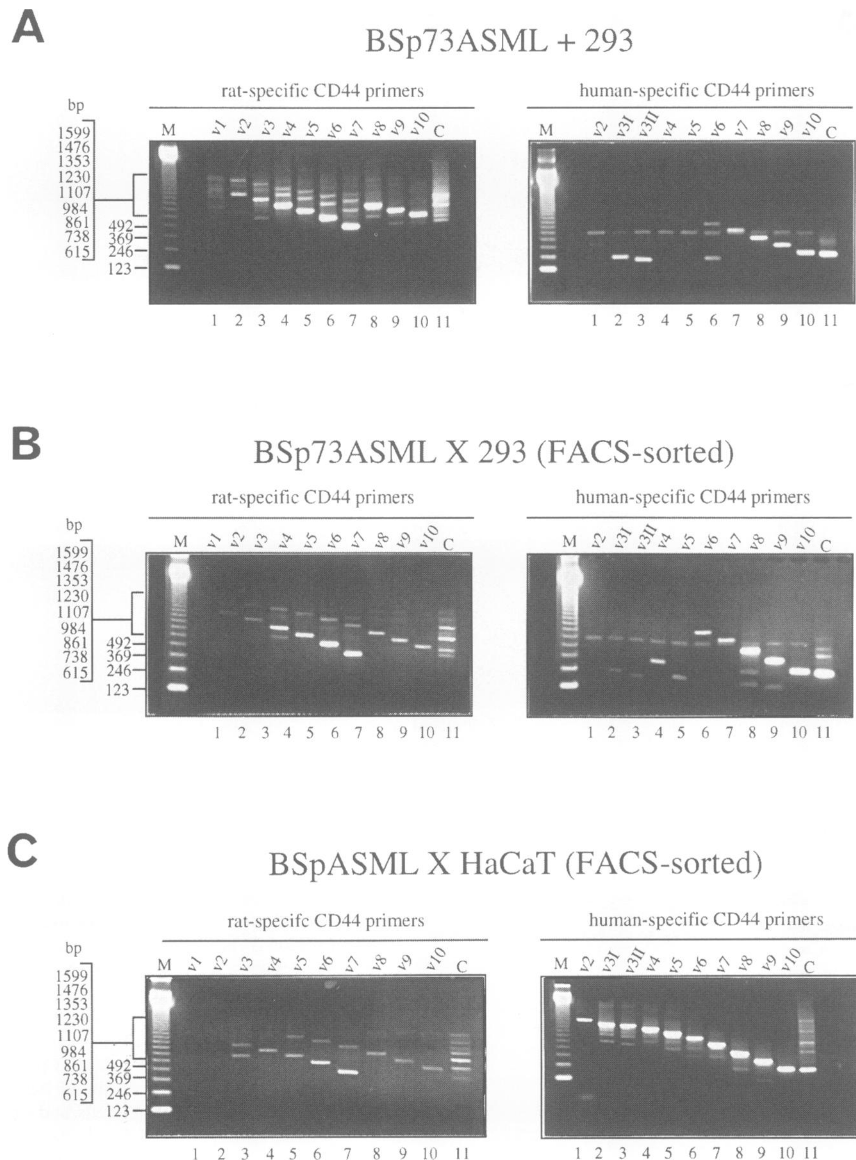

Fig. 5. Exon-specific RT–PCR analysis of mixed cell populations of rat BSp73ASML carcinoma cells and human 293 embryonal kidney cells, BSp73ASML+293 (**A**) and heterokaryons enriched by FACS, BSp73ASML×293 (**B**). (**C**) Exon-specific RT–PCR analysis of heterokaryons of human HaCaT keratinocytes and rat BSp73ASML carcinoma cells (BSp73ASML×HaCaT) enriched by FACS. RNA was prepared 5 h after cell fusion. PCRs were performed using either human-specific (hs5′ and hs3′; right panels) or rat-specific (rs5′ and rs3′; left panels) CD44 primers for pre-amplification. Exon-specific PCRs were performed using the 5′ primers specific for the variant exons indicated (lanes 1–10) and the constant region 5′ primer C13 (C, lane 11), respectively, in combination with the 3′ rat C2A primer or the 3′ human primer hs3′. For primer positions, see Figure 3. Amplification products were resolved on ethidium bromide-containing agarose gels and visualized by UV irradiation. M, DNA size marker (123 bp ladder, Life Technologies Inc.). Numbers on the left indicate marker sizes in bp. Asterisks denote an unspecific PCR product.

mammary carcinoma line and in the human cervical carcinoma cell lines SiHa and ME180 (not shown).

Discussion

Somatic cell hybrids (synkaryons) can be used to investigate positive and negative regulatory influences when genetic materials from two cells of distinct differentiation states are included in the same cytoplasm (Ephrussi, 1972; Davidson and de la Cruz, 1974). Here we asked whether a differentiation-specific splice pattern behaves as a dominant or recessive trait upon fusion of cells with different splicing patterns. In the stable somatic cell hybrids between cells expressing CD44s and those expressing CD44v, the expression of CD44 variants was not extinguished, indicating a dominant mechanism for variant exon expression. Also, transient interspecies heterokaryons which do not suffer from the problem of chromosome loss (Baron and Maniatis, 1986) and which allow one to distinguish between *cis*- and *trans*-acting mechanisms, confirmed the existence of a dominant mechanism. Furthermore, they indicated the existence of *trans*-acting factors inducing the inclusion of CD44 variant exon sequences. Several CD44 variant-expressing tumor cell lines also possess *trans*-acting factors involved in CD44 variant expression. In one particular rat pancreatic carcinoma cell line (BSp73ASML), however, one allele seems to carry a *cis*-acting mutation, in that the expression of splice products ending with exon v7 in the variant region (e.g. CD44v4–v7 and CD44v6–v7) can neither be altered by factors from another nucleus nor be transferred.

Several mechanisms are known which can affect splice site selection in constitutively or alternatively spliced pre-mRNAs. Both intron and exon sequences of the pre-mRNA can influence regulated splice site selection (Libri *et al.*, 1990; Black, 1991, 1992; Nemeroff *et al.*, 1992; Watakabe *et al.*, 1993, and references therein). Exon sequences can either prevent the use of adjacent splice sites (Gallego *et al.*, 1992; Nemeroff *et al.*, 1992; Siebel *et al.*, 1992) or can stimulate splice site usage (Reed and Maniatis, 1986; Hampson *et al.*, 1989; Streuli and Saito, 1989; Libri *et al.*, 1990; Cooper, 1992; Cote *et al.*, 1992; Graham *et al.*, 1992). Purine-rich sequence elements, so-called exonic splice enhancers, have been identified in both mammals and *Drosophila* (Lavigueur *et al.*, 1993; Watakabe *et al.*, 1993; Xu *et al.*, 1993; Dirksen *et al.*, 1994; Tanaka *et al.*, 1994; Lynch and Maniatis, 1995). Moreover, in the final step of the sex determination pathway in *Drosophila*, proteins (Tra and Tra-2) have been identified that interact with such splice enhancer sequences in the *doublesex* pre-mRNA and thereby stimulate the utilization of the upstream 3′-splice site of the female-specific *doublesex* exon (Hedley and Maniatis, 1991; Hoshijima *et al.*, 1991; Tjian and Maniatis, 1993). In mammals, several factors that can affect 5′ splice site selection *in vitro* and *in vivo* have been cloned (reviewed in Horowitz and Krainer, 1994). Most of them, like the *Drosophila* Tra proteins, belong to the SR family of proteins (Zahler *et al.*, 1992). However, the pre-mRNA and exon specificity as well as the cell type specificity of their expression and the mode of action of SR proteins are not clear. Thus, in most cases, sequences, factors and the mechanisms regulating alternative splicing are still not known.

Alternative splicing of CD44 pre-mRNA is particularly interesting because of the large variability derived from the choice of 10 variant exons. This study permitted us to make the following conclusions. (i) The *trans*-acting splicing regulators involved in CD44 alternative splicing act positively in terms of inducing the inclusion of variant CD44 exon sequences, suggesting that skipping of variant exon sequences in most tissues is the default pathway for CD44 mRNA splicing. (ii) Obviously, these factors are conserved between mammalian species and determine the splicing pattern of CD44 pre-mRNA in a cell-type specific manner. (iii) A preferred mechanism of variant exon recruitment seems to be directional, starting with v10 and including exons in the 5′ direction. (iv) Progressive inclusion of variant exons in the 3′ (v10) to 5′ (v1) direction does not explain all splice patterns transferable by cell fusion. Rather, factors must exist which recruit individual exon sequences. Such factors would account for the expression of single-exon variants, e.g. CD44v3 in the 293 human embryonal kidney cells, and for the transfer of specific recruitments to another nucleus, e.g. CD44v4–v5 and CD44v5 induced in the 293 nuclei as distinct variants rather than as part of larger variants containing 5′ exons when 293 cells were fused with the CD44v4–v7-expressing BSp73ASML rat carcinoma cells.

Interestingly, in the case of CD45, a receptor-type protein tyrosine phosphatase, the existence of negative *trans*-acting factors repressing the inclusion of alternative exons into CD45 mRNA has been demonstrated (Rothstein *et al.*, 1992). Thus, in mammalian cells (e.g. lymphocytes) different mechanisms seem to exist by which *trans*-acting splice factors can regulate alternative splicing of distinct genes. In fact, the two mechanisms described appear to work simultaneously in activated T lymphocytes. Following antigenic stimulation, human T cells down-regulate CD45RA expression by splicing out alternative exons resulting in CD45RO expression (Deans *et al.*, 1989). In contrast, variant exons are included in CD44 mRNA during T-cell activation (Arch *et al.*, 1992; Koopman *et al.*, 1993). In addition, CD45RO and CD44v6 were shown to be co-expressed on T cells following activation by phytohemagglutinin (Screaton *et al.*, 1995). These findings indicate that pre-mRNAs of distinct genes can be regulated differently with respect to skipping or retention of exons within the same cell. The changes in CD44 and CD45 splicing following T-cell activation have been shown to coincide with changes in mRNA levels of certain SR proteins (Screaton *et al.*, 1995). Whether this is also accompanied by changes in SR protein levels and whether there is a causal relationship to the changes observed in CD44 and CD45 splicing is, however, not clear. Exon skipping versus exon inclusion in different pre-mRNAs within the same cell suggests the existence of distinct factors which are able to recognize specifically pre-mRNA elements of different genes or sets of genes. Therefore, a situation similar to that of transcriptional regulators can be envisaged, in which a multitude of splice regulators with different pre-mRNA and exon binding specificities would allow the differential expression of protein isoforms from the same or from distinct genes.

Materials and methods

Cell lines and cell culture

HaCaT is a spontaneously immortalized human keratinocyte cell line (Boukamp *et al.*, 1988); BSp73ASML, a metastatic rat pancreas carcinoma cell line (Matzku *et al.*, 1983); MTLn3, a metastatic rat mammary carcinoma cell line (Neri *et al.*, 1982); NIH3T3, a mouse embryonal fibroblast cell line (ATCC CRL1658); KLN205, a mouse carcinoma cell line (ATCC CRL1453); Rat2, a rat embryonal fibroblast cell line (ATCC CRL1764); 293, a human embryonal kidney cell line (ATCC CRL 1573); and SiHa (ATCC HTB35) and ME180 (ATCC HTB33) are human metastatic cervical carcinoma cell lines.

All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, at 37°C and 6% CO_2.

Cell fusion

For cell fusions, adherent cells were trypsinized, washed twice in serum-free DMEM and equal numbers of cells ($0.5–1\times10^7$) were mixed and pelleted by centrifugation. After complete removal of the medium, cells were fused by stirring with 50% PEG in phosphate-buffered saline (PBS) without Ca^{2+} (Sigma) as described (Harlow and Lane, 1988).

Generation and analysis of stable synkaryons

For generation of stable synkaryons, NIH3T3 and KLN205 cells were stably transfected with the plasmids pSV2neo (Southern and Berg, 1982) and pGEMpuro (expresses the puromycin *N*-acetyl transferase gene under the control of the phosphoglycerate kinase promoter; K.Kondo, unpublished), respectively, by lipofection. Resistant clones were selected in the presence of either 800 µg/ml G418 (GIBCO BRL) or 1.5 µg/ml puromycin (Sigma), and the resulting clones (>100 each) were pooled. Cells were fused by PEG as described before and stable synkaryons were selected in medium containing 800 µg/ml G418 and 1.5 µg/ml puromycin.

For determination of the DNA content, the cells were fixed in 70% ethanol, stained by propidium iodide as described (Noguchi, 1991) and analyzed by flow cytometry analysis using a FACStar Plus (Becton and Dickinson). CD44 expression was determined by flow cytometry analysis

Table I. Primers used for RT–PCR and exon-specific PCRs[a]

Primer	Sequence
hs5′	5′-GATGGAGAAAGCTCTGAGCATC-3′
hs3′	5′-TTTGCTCCACCTTCTTGACTCC-3′
Human C13	5′-AAGACATCTACCCCAGCAAC-3′
Human pv2	5′-GATGAGCACTAGTGCTACAG-3′
Human pv3I	5′-ACGTCTTCAAATACCATCTC-3′
Human pv3II	5′-TGGGAGCCAAATGAAGAAAA-3′
Human pv4	5′-TCAACCACACCACGGGCTTT-3′
Human pv5	5′-GTAGACAGAAATGGCACCAC-3′
Human pv6	5′-GAGGCAACTCCTAGTAGTAC-3′
Human pv7	5′-CAGCCTCAGCTCATACCAGC-3′
Human pv8	5′-TCCAGTCATAGTACAACGCT-3′
Human pv9	5′-CAGAGCTTCTCTACATCACA-3′
Human pv10	5′-GGTGGAAGAAGAGACCCAAA-3′
rs5′	5′-CGACCCTTTTCCAGAGGCGACTA-3′
rs3′	5′-CGTCTCCAATCGTGCTGTCTTTTC-3′
Rat C13	5′-AAGACATCGATGCCTCAAAC-3′
Rat C2A	5′-GGCACTACACCCCAATCTTC-3′
Rat pv1	5′-GCCTCAACTGTGTACTCAAA-3′
Rat pv2	5′-GATGACTACCCCTGAAACAC-3′
Rat pv3	5′-ACGGAGTCAAATACCAACCC-3′
Rat pv4	5′-TGCAACTACTCCATGGGTTT-3′
Rat pv5	5′-TATAGACAGAAACAGCACCA-3′
Rat pv6	5′-TGGGCAGATCCTAATAGCAC-3′
Rat pv7	5′-CTGCCTCAGCCCACAACAAC-3′
Rat pv8	5′-CCAGTCATAGTACAACCCTT-3′
Rat pv9	5′-CAGAACTTCTCTACATTACC-3′
Rat pv10	5′-GGTCGAAGAAGAGGTGGAAG-3′

[a]For primer positions, see Figure 3.

after staining the cells by the mAb IM7 (rat anti-mouse CD44s; ATCC) for overall CD44 expression (CD44pan) and the mAB 10D1 (rat anti-mouse CD44v4; obtained from J.Sleeman; unpublished) for expression of CD44v4. As second antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rat immunoglobulins (Igs) were used.

Transient heterokaryons and fluorescence-activated cell sorting (FACS)

For the generation of transient heterokaryons, 1×10^7 cells of each cell type were fused as described above and replated on tissue culture dishes for 5 h before cells were harvested for flow cytometry analysis, FACS and RNA preparation. For flow cytometry analysis, cells were stained with the rat anti-mouse CD44 mAb IM7 (cross-reacts with human but not with rat CD44; our own unpublished observations) and the mouse anti-rat CD44 mAb Ox50 (recognizes specifically rat CD44 in all isoforms; ATCC). Cells bound by these mAbs were then detected by FITC-conjugated anti-rat Ig and R-phycoerythrin (RPE)-conjugated anti-mouse Ig, respectively. CD44v6 expression was analyzed by staining the cells in a third step by biotinylated mouse anti-rat CD44v6 mAb 1.1 ASML (Günthert *et al.*, 1991) which was detected by cychrome-coupled streptavidin. FACS was performed using a FACStar Plus (Becton and Dickinson) and 1×10^4–7×10^4 FITC and RPE double-fluorescent cells were obtained for RNA preparation.

RT–PCR and exon-specific PCR analyses

Cellular RNA for RT–PCR was prepared as described (Chomczynski and Sacchi, 1987). Reverse transcription was performed with 5 U of AMV reverse transcriptase (Promega) in 20 μl reactions containing 50 mM Tris–HCl pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 5 mM spermidine and 20 U RNasin (Promega) using 500 ng of RNA and 100 ng of oligo $d(T)_{15}$ primer (Boehringer Mannheim). Reactions were incubated for 1 h at 41°C. Following heat-inactivation of the reverse transcriptase for 10 min at 65°C, the volume was increased with water to 200 μl. PCRs were performed in a microprocessor-controlled incubator (Crocodile II, Appligene) using 5 μl of the diluted reverse transcriptase reactions in a reaction volume of 50 μl containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl pH 9.0, 250 μM dNTPs and 2.5 U of *Taq* DNA polymerase. Twenty-two cycles were carried out for GAPDH and 30 cycles for CD44 amplifications, respectively, each consisting of 1 min at 94°C, 1 min at 59°C (GAPDH primers and rat-specific CD44 primers) or 56°C (human-specific CD44 primers) and 2 min at 72°C. Amplification products were resolved on 1% agarose gels, blotted to nylon membranes (Hybond-N+, Amersham) and hybridized to ^{32}P-labeled CD44 cDNA probes encompassing either the whole CD44 standard cDNA or exon sequences v1–v3.

Pre-amplification conditions for exon-specific PCRs were as follows. Using rat-specific primers (rs5′ and rs3′): 20 cycles each consisting of 60 s at 94°C, 60 s at 56°C and 90 s at 72°C. Using human-specific primers (hs5′ and hs3′): 25 cycles each of 60 s at 94°C, 60 s at 56°C and 90 s at 72°C. Exon-specific PCRs were performed in 50 μl reactions as described above using 1.5 μl of the pre-amplification reactions. Rat CD44 cDNAs were amplified using the rat C13 and the exon-specific rat primers pv1–pv10 as 5′ primers and C2A as a 3′ primer, performing 25 cycles of 60 s at 94°C, 60 s at 54°C and 90 s at 72°C. Human CD44 cDNAs were amplified using the human C13 and the exon-specific human primers pv2–pv10 as 5′ primers and the primer hs3′ as a 3′ primer, performing 22 cycles of 60 s at 94°C, 60 s at 56°C and 90 s at 72°C. Amplification products were resolved on 2% agarose gels containing 0.3 μg/ml ethidium bromide. The primers used in the RT–PCR and the exon-specific PCRs are listed in Table I.

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