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VARIANT CD44 SURFACE PROTEINS, DNA SEQUENCES CODING THEM, ANTIBODIES AGAINST THESE PROTEINS AND THEIR USE IN DIAGNOSIS AND THERAPY

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(57) Claim

1. DNA fragment, which codes for a part of a surface protein of a metastasising ~~metastasing~~ tumour cell, whereby this DNA fragment is selected from

a) the nucleotide sequences

...rMeta-1...

ATT GCA ACT ACT CCA TGG GTT TCT GCC CAC ACA AAA CAG AAC
CAG GAA CGG ACC CAG TGG AAC CCG ATC CAT TCA AAC CCA GAA
GTA CTA CTT CAG ACA ACC ACC AGG ATG ACT GAT ATA GAC AGA
AAC AGC ACC AGT GCT CAT GGA GAA AAC TGG ACC CAG GAA CCA
CAG CCT CCT TTC AAT AAC CAT GAG TAT CAG GAT GAA GAG GAG
ACC CCA CAT GCT ACA AGC ACA ACC TGG GCA GAT CCT AAT AGC
ACA ACA GAA GAA GCA GCT ACC CAG AAG GAG AAG TGG TTT GAG
AAT GAA TGG CAG GGG AAG AAC CCA CCC ACC CCA AGT GAA GAC
TCC CAT GTG ACA GAA GGG ACA ACT - GCC TCA GCC CAC AAC
AAC CAT CCA AGT CAA AGA AGT ACA ACA CAG AGT CAA GAG GAT
GTT TCA TGG ACC GAT TTC TTC GAC CCA ATC TCA CAT CCA ATG
GGA CAA

and

.../2

hMeta-1...

AAC CCA AGC CAT TCA AAT CCG GAA GTG CTA CTT CAG ACA ACC
ACA AGG ATG CAT GAT GTA GAC AGA AAT GGC ACC ACT GCT TAT
GAA GGA AAC TGG AAC CCA GAA GCA CAC CCT CCC CTC ATT CAC
CAT GAG CAT CAT GAG GAA GAA GAG ACC CCA CAT TCT ACA AGC
ACA ATC CAG GCA ACT CCT AGT AGT ACA ACG GAA GAA ACA GCT
ACC CAG AAG GAA CAG TGG TTT GGC AAC AGA TGG CAT GAG GGA
TAT CGC CAA ACA CCC AGA GAA GAC TCC CAT TCG ACA ACA GGG
ACA GCT GCA GCC TCA GCT CAT ACC AGC CAT CCA ATG CA

b) nucleotide sequences which encode the same peptide encoded by the DNA sequences mentioned under a) above;

c) nucleotide sequences which encode a surface protein representing allelic variations of the surface peptide encoded by the DNA sequences mentioned under a) above; or

d) any other nucleotide sequence which encodes a peptide with conservative amino acid changes, and having the biological function of the peptide encoded by the DNA sequences mentioned in a), b) or c) above.

11. Use of a DNA fragment according to claim 1, its complementary strand, derivatives and parts thereof for the identification, production or isolation of a nucleotide sequence or parts thereof which code for a part of a surface glycoprotein of a metastasising tumour cell.

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(54) Title: VARIANT CD44 SURFACE PROTEINS, DNA SEQUENCES CODING THEM, ANTIBODIES AGAINST THESE PROTEINS AND THEIR USE IN DIAGNOSIS AND THERAPY

(54) Bezeichnung: VARIANTE CD44-OBERFLÄCHENPROTEINE, DIESE KODIERENDE DNA-SEQUENZEN, ANTIKÖRPER GEGEN DIESE PROTEINE SOWIE IHRE VERWENDUNG IN DER DIAGNOSTIK UND THERAPIE

(57) Abstract

The invention relates to variant CD44 surface proteins. Antibodies against the variant determinant of these proteins and a process for producing them, and in addition the C-DNA sequences which code for these variant protein pieces, and the use of these proteins or parts thereof and the antibodies directed against them for the diagnosis and treatment of tumor metastases.

(57) Zusammenfassung

Die Erfindung betrifft variante CD44-Oberflächenproteine. Antikörper gegen die variante Determinante dieser Proteine sowie Verfahren zu ihrer Herstellung, außerdem die C-DNA Sequenzen, die für diese varianten Proteinstücke kodieren, sowie die Verwendung dieser Proteine oder Teile davon und die gegen sie gerichteten Antikörper zur Diagnostik und Therapie von Tumormetastasen.

Variant CD44 surface proteins, DNA sequences coding these, antibodies against these proteins, as well as their use in diagnosis and therapy

The invention concerns variant CD44 surface proteins, antibodies against the variant determinants of these proteins, as well as processes for their production, furthermore the DNA sequences which code for these variant protein fragments, as well as the use of these proteins or parts thereof and the antibodies directed against them for the diagnosis and therapy of tumour metastases.

The ability to ~~metastase~~ metastasise forms the actual life-endangering property of malignant tumour cells. The original primary tumour cells probably acquire this property by a whole series of changes in the course of the tumour progression. As a result of this process, cancer cell variants are continuously detached from the primary tumour mass, penetrate the extracellular matrix and migrate into the lymphatic system or the blood circulation. Often adhering to one another, the ~~metastasing~~ metastasising tumour cells are transported in the blood or lymph system, leave the vascular system at other places in order there to penetrate into secondary tissue and form daughter tumours (survey of Hart et al., 1989; Nicolson, 1987). The formation of metastases requires a whole series of interactions of the tumour cells with intercellular matrix and other cells. Almost all of these interactions require cell surface components, such as e.g. the receptors for matrix and lamina, surface-bound proteolytic enzymes, as well as cell adhesion molecules with inclusion of those which cause organ-specific adhesion and thus organ preference of the metastasis, furthermore growth factors and growth factor receptors.

It is known that the membrane proteins differentiate non-~~metastasing~~ metastasising and ~~metastasing~~ metastasising tumour cells of the

BSp73 rat tumours, demonstrated by antibody reaction (Matzku et al., 1983 and 1989).

It has now been found that the metastasing BSp73ASML tumour cells contain a surface protein which, in part, corresponds to a known glycoprotein participating in the lymphocyte adhesion and cell-cell and cell-matrix exchange action (designation of the normal glycoprotein in humans: CD44, hermes-1, in the mouse: Ppg-1 and in the rat: HEBFln). However, the new variant CD44 surface protein differs from these known sequences by an extracellular region (ECR) of 154 amino acids which is introduced between the 220th and 237th amino acid of the human CD44 sequence (or 224th and 239th amino acid of the mouse sequence). This new glycoprotein appears to possess an important role for the cell/matrix or cell/cell binding in the case of the metastasis. Therefore, the production and characterisation of this protein region (ECR) forms one of the tasks of the present invention. By immunisation of mice with membrane proteins which have been obtained from BSp73ASML, spleen cells were produced which form antibodies against the ECR of the variant CD44 surface protein. According to the method of Köhler (1981), these are fused by polyethylene glycol with myeloma cells in order to produce permanent cultures. By means of cloning and selection of those cultures which produce antibodies which react with BSp73ASML but not with the non-~~metastasing~~ metastasising parent form and also not with other non-tumorigenic rat cells, there can be obtained specific antibodies against the new protein part ECR. For the further investigation, a monoclonal antibody was chosen which stains the BSp73ASML cells in the immunofluorescence test especially intensively, which has received the designation mAb1.1ASML (mAb: monoclonal antibody).

In the Western blot test, in a protein hydrolysate from BSp73ASML, there can be determined 4 protein bands

with molecular weights of 120,000; 150,000; 180,000 and 200,000 with mAb1.1.ASML, whereas extracts from rat fibroblast cells and non-metastasing rat tumour cells give no significant reaction. It has not yet been possible to determine whether these size differences are due to a different original amino acid sequence or to a differently strong subsequent protein modification. In any case, the epitope recognised by the antibody is contained in all 4 protein species but not in the proteins serving for the control from the non-metastasing BSp73As cells or from normal rat cells.

Isolation of cDNA sequences which code for the ECR of the surface protein

The monoclonal antibody mAb1.1ASML was used in order to discover the ECR-coding cDNA sequences in a bacterial expression bank. The bank was constructed with the help of PolyA+ RNA from BSp73ASML and the pEX vector system (Stanley & Luzio, 1984). The products coded by the cDNA sequences are found as β-galactosidase fusion proteins with the help of the antibodies. A so-isolated cDNA clone positive for the monoclonal antibody 1.1ASML with the name pEX34 carries a cDNA sequence piece of 167 nucleotides. This cDNA piece was now used in order to pattern through a larger cDNA bank in the vector pSP65, again from BSp73ASML RNA. One of these clones isolated therewith, pM66, served thereto to isolate the total length cDNA clone pMeta-1 with the help of so-called "primer" elongation (starter oligo-nucleotides) and of the polymerase chain reaction (PCR). Evidence of the total length was obtained with the help of the primary elongation (3207 nucleotides). The colinearity with an RNA from the BSp73ASML tumour was documented by RNase and S1 protection analysis.

The isolation of the cDNAs with the help of the expression in bacteria and the recognition by the antibodies proved that the antibody recognised primary amino

acid sequence in the ECR of the surface protein.

ECR-coding messenger RNA is expressed in BSp73ASML but not in BSp73AS cells.

Three different sequence samples were produced from the cDNA clones in order to demonstrate specific messenger RNAs by hybridisation. A sample A covers the cDNA region which codes for the ECR (positions: 941 - 1108). The sample B represents sequences between the positions 1403 - 1572 and sample C carries sequences from the start up to position 794 from pMeta-L (Fig. 1). Poly A+ RNA of the BSp73 tumour cell line was separated electrophoretically and analysed with the help of RNA transfer hybridisation. Four of the cell lines, which do not metastase, contain no RNA which is homologous to the sample A, whereas RNAs from the ~~metastasing~~ metastasising tumour cells strongly react BSp73ASML. In this RNA preparation, the sample A recognises a heterogenous mixture of various RNA sizes between 2.2 and 3.3 kb and a larger RNA species of 4.9 kb. The exclusive expression of the specific membrane proteins recognised by the monoclonal antibody 1.1ASML in the metastasing tumour cell variant is obviously based upon the exclusive expression of the corresponding ECR-coding messenger RNAs. Obviously the complete cDNA clone pMeta-1 with 3.2 kb cannot represent all sequences of this RNA species. It can only represent one species from the heterogenous mixture of RNA sizes. The samples B and C give the same hybridisation pattern as sample A in the separation of the BSp73ASML RNA in any case as far as one can ascertain in the heterogeneity, i.e. these RNA species carry sequences which are complementary to all three samples, A, B and C. In contradistinction to sample A, sample B and C also recognise messenger RNA species in the non-~~metastasing~~ metastasising cell lines. However, the RNA sizes differ clearly from those in BSp73ASML, there are, namely, detected four clearly differentiatable

messenger RNA species with the sizes 1.5, 2.0, 2.9 and 4.3 kb. Although these RNA species could be hidden in the heterogenous mixture of the RNAs from BSp73ASML, it is, nevertheless, certain that they do not exist in the same amount in the BSp73ASML. It is decisive that RNA sequences with complementarity to sample A are obviously completely absent in the non-~~metastasing~~ metastasising cells. Therefore, we can carefully conclude that the sequences of the samples A, B and C are contained in the same RNAs in the ~~metastasing~~ metastasising tumour, namely, in a manner as though sample A sequences have been spliced into the B-C-positive RNAs and as though this alternative splicing process only occurred in the ~~metastasing~~ metastasising cell line.

In order to demonstrate the colinearity between RNA and cDNA and in order to analyse the difference of the RNAs between the BSp73AS and the BSp73ASML cells, S1 nuclease and RNase protection analyses were carried out. The protected DNA or RNA fragments could only be smaller than the total length because they contain 5'-end vector sequences which cannot hybridise with the RNAs from the tumour cells. We consider first the transfer to the 3' side: the transfer of sequences with homology to sample A to those to sample B. Both techniques show a single RNA species in BSp73AS which is colinear with the samples over a wide range. Furthermore, 5' thereof differentiate cDNA or RNA sample, which certainly correspond to RNA sequences from BSp73ASML, from the RNA from the BSp73AS cells. In particular, the RNAs from BSp73ASML contain sequences which protect larger fragments of the samples. The largest fragments correspond to the full length of the DNA or RNA pieces which were offered for the protection analysis. Smaller fragments are also detectable. Since the RNA transfer hybridisations have certainly uncovered a heterogenous mixture of different

sizes of RNAs, it is possible that these indicate smaller protected fragments of RNA species which diverge elsewhere from the cDNA, i.e. at positions between the previously detected divergence point and the 5' end of the offered samples. The RNA species are also not detectable in the BSp73AS cells.

We now consider the point of the divergence on the 5' side, thus the transition of sequences which hybridise with the sample C to those which hybridise with the sample A, thus the ECR-coding sequence. The analysis gives corresponding results for the 5' breaking point. RNAs from BSp73AS can protect the offered samples only over a small range. Messenger RNAs from BSp73ASML protect longer fragments. They are, namely, colinear over the whole length of the offered sample. One can thus conclude that the cDNA clone represents pMeta-1 sequences which are distinctive in the ~~metastasing~~ metastasising tumour cells BSp73ASML. The 3' and 5' regions are also found in RNAs from BSp73AS. The ECR-coding sequences with the definite transitions, which can be mapped with the help of these above-described techniques, indicate that here an alternative splicing mechanism must be present for the RNA formation. The 5' and 3' breakage points of the transitions to the ECR sequences are marked by arrows in Fig. 1.

<u>The monoclonal antibody 1.1ASML identifies a variant form of the glycoprotein CD44</u>.

In order to obtain structural information about the surface protein, all cloned cDNA molecules have been sequenced. The nucleotide sequence of the total length clone pMeta-1 and the amino acid sequences derived therefrom are shown in Figure 2. The total length cDNA clone traverses 3207 nucleotides. The 3' terminus carries a PolyA end, two additional polyadenylation signals lie at positions 2288 and 1743. The first ATG

codon follows a consensus initiation sequence and opens a reading frame of 1509 nucleotides, corresponding to 503 amino acids. As one should assume for a membrane-standing protein, the first 21 amino acids are hydrophobic and represent a signal peptide. No part of these sequences is hitherto to be found in the data bases. However, we found sequence homology to the recently published data about the lymphocyte homing receptor CD44 (of Pgp-1) (Idzerda et al., 1989; Goldstein et al., 1989; Stamenkovic et al., 1989; Nottenburg et al., 1989; Zhou et al., 1989). The homologies are strictly limited to the 5' and 3' parts of the cDNA with inclusion of non-translated regions and they end at the already above-mentioned points of divergence between the BSp73AS and the BSp73ASML RNA sequences. The total extent between the divergence points (in Figure 2 characterised by colour markings), thus the whole extent of the metastasis-specific ECR-coding sequence, is not represented in the Pgp1 or CD44 sequences. The metastasis-specific glycoprotein obviously represents a variant of the CD44 glycoproteins. It carries, namely, an additional extracellular domain of 156 amino acids and thus an expanded extracellular region of 410 amino acids (less 21 amino acid signal peptide), in comparison with 270 amino acids (also less signal peptide) of the unchanged CD44 glycoprotein. However, in the non-~~metastasing~~ metastasising BSp73AS cells, the unchanged forms of this CD44 family are detected. cDNA sequences of these BSp73AS RNAs have also been cloned and the identity with the metastasis-specific clones outside of the extra domain is demonstrated.

The expression of the variant CD44 is correlated with the metastatic potential.

In order to test whether the expression of the variant CD44 glycoproteins takes place without exception

in the BSp73ASML cells and whether it stands in connection with the metastatic potential of these cells or with the metastatic potential in general, we studied a series of isogenic rat tumour cell lines, namely, the tumour cell lines of the mammary carcinoma system 13762 NF (Neri et al., 1982). We here compare cell lines which have been derived from the parental tumour, namely, the MTPa, MTC, MTF7 and MTA cells (group 1), with cell lines which were established from lymph nodes or lung metastases, namely, MTLy, MTLn2, MTLn3 (group 2). The group 1 cells essentially express the normal CD44 pattern similarly to the RNAs from the BSp73AS cells when one hybridises with sample B. On the other hand, with sample A, there is detected a smaller amount of a diffuse RNA band which has about the size 2.5 kb. On the other hand, the group 2 cells show a completely different RNA pattern. Both samples A and B hybridise with larger RNA species. The sizes resemble those which are detected with BSp73ASML. The similarity is also documented by RNAse and S1 protection analyses. On the basis of these data, we conclude that a change of the splicing pattern of the RNA and the expression of variant CD44 is correlated with the formation of metastases and that the acquired pattern in these ~~metastasing~~ metastasing mammary carcinoma cells corresponds very much to those which we have already got to know for the metastasing BSp73ASML cell line. The high molecular proteins recognised by the antibodies correspond to the two high molecular species of proteins which were detected in the BSp73ASML extracts. In this mammary tumour series, we thus discovered also a metastasis-specific expression of RNA species and of high molecular proteins. That in the group 1, thus the so-called parenteral cell lines, any RNAs were found at all which hybridised with the sample A, thus the ECR-coding sequence, and that we can also see a weak coloration

of a protein of 100,000 Dalton with the antibody, we attribute to the fact that the group 1 cells also possess small ~~metastasing~~ ability quite contrary to our original cell line BSp73AS which shows no metastasing behaviour at all.

The monoclonal antibody 1.1ASML inhibits metastasis formation in the rat.

In a series of experiments for the metastasis formation of the tumour cell line BSp73ASML in isogenic rats, cells were injected subcutaneously and at different times the monoclonal antibody 1.1ASML was injected intraperitoneally at intervals of two to three days before and after the tumour administration. In the scope of this immunological protocol, it was also determined how the immune response of the rat towards the injected antibody had taken place. There result, namely, anti-mouse immunoglobulin antibodies, as well as also anti-idiotype antibodies. The result of this series of experiments is that the growth and the metastasing of the tumour is considerably delayed by injection of 1.1ASML. This delay permits, in its kinetic, the conclusion that the antibody interferes with a primary process of the ~~metastasing~~ metastasising. The experiment shows to us that the protein structure on the surface of the ~~metastasing~~ metastasising cells recognised by the antibody has a role in the ~~metastasing~~ metastasising process and that therapeutic and diagnostic plans are realistic.

Isolation of the homologous human sequence for the ECR-coding sequence part of the rat cDNA.

For human tumour cells in culture, the possibility naturally does not exist as a matter of course of detecting, correspondingly the rat system, whether they also still retain ~~metastasing~~ metastasising properties. Experiments with immune-deficient mice make possible only very limited predictions regarding the ~~metastasis~~ metastatic potential in the case of humans. Therefore, relatively many

tumour cell lines, which have been taken up in culture anywhere in the world at points of time lying a long time ago, would have to be tested for whether they express the sequences which we could detect for the rat metastases. It has been possible to find such a tumour cell line. It originates from a large-cell lung carcinoma of humans and bears the number: LCLC97. In this tumour cell line can be detected three definite RNA species (sizes: 5.5; 3.4 and 2.8 kb) which behave quite corresponding to the RNAs which are detectable in the ~~metastasing~~ metastasising tumour cell lines of the rat. They hybridise, namely, not only with the sample A but also with the samples B and C, i.e. that also these human RNA species are identical over wide ranges to the cDNA pMeta-1 (85%).

However, the monoclonal antibody 1.1ASML does not react with this tumour cell, i.e. the piece of protein recognised by the antibody must, in the region of the antigen determinant, differ from the proteins which exist on the surface of the human tumour cells. For the non-reactivity, there suffice already the smallest variations on the basis of the high specificity of the antibody. The human tumour cell LCLC97 now served the purpose of constructing a cDNA bank. On the basis of the high agreement between the rat and human sequences, a cDNA clone could be isolated which showed homology with the sample A. The human cDNA was sequenced. In Figure 3 (a,b) is shown the primary sequence and the amino acid sequence derived therefrom. One can see that, over large regions, identicity exists between the rat and the human sequence. This human sequence, as well as also the amino acid sequence derived therefrom, is also the subject of this Patent Specification.

Embodimental examples:

Cells and antibodies:

The following cloned Bsp cell lines were used for the investigation: BSp73 14ASML-1 and 10AS-7 and kept in culture as described by Matzku et al., (1983); furthermore, the mammary carcinoma cell lines described by Neri et al., (1982), monoclonal antibodies against BSp73 ASML membrane proteins were produced by immunisation of Balb c mice. After isolation of the spleen cells of an immunised mouse, these were fused with Ag8 myeloma cells for the immortalisation according to the method for the production of monoclonal antibodies of Köhler (1981). The then-obtained hybridoma cells were subjected to a screening process in order to find those which produce specific antibodies against BSp73ASML but not against BSp73AS and normal rat fibroblast cells. The precise procedure is described in the same way as by Matzku et al. (1989).

Monoclonal antibody (mAb)-producing hybridoma cells with the corresponding specificity were expanded in the tissue culture and the mAb given off into the medium highly enriched by ammonium sulphate precipitation and column chromatography (protein A-Sepharose and MonoQ) and used in this form for the investigations. One of them is mAb1.1ASML.

Immunofluorescence:

For the display of the variant CD44 molecule on different tumour cells, these were taken up in culture, then washed with phosphate-buffered common salt solution (PBS) and incubated with 1.1ASML for 30 minutes at 40°C. As secondary antibody for the detection of the binding, there was used a rhodamine-coupled rabbit anti-mouse IgG and shown in the fluorescence microscope.

Construction of the cDNA expression banks and immuno-screening.

PolyA+ RNA from BSp73ASML cells was "primed" with

oligo (dT) and hexanucleotides of different composition and synthesised with reverse transcriptase from AMV of the first strand of the cDNA. The second strand of the cDNA was produced with E. coli DNA polymerase I, RNaseH and E. coli ligase and subsequently the double-stranded cDNA linearised on the ends with T4DNA. The vectors pEX1, 2 and 3 (Stanley and Luzio, 1984), which make possible the fusioning of the cDNA in 3 different reading rasters, were cleaved with SmaI restruction endonuclease and ligated with the cDNA (T4 DNA ligase). Competent E. coli DH5 (pCI857) bacteria, which produce a temperature-sensitive repressor, are transfected with the pEX-cDNA constructants and cultured on nylon filters. The gene for the temperature-sensitive repressor RCI857 lies on the plasmid pCI857, which is compatible with the pEX plasmids. At 28°C, the $1P_R$ promotor, which controls the synthesis of the fusion proteins, is inactivated. By temperature increase to 42°C, the CI repressor is inactivated and the synthesis of β-galactosidase/ASML fusion proteins massively set into action. The heat-induced bacteria colonies are subsequently denatured with chloroform vapour on the filters and these then incubated in PBS which contains 3% dry milk powder, lysozyme and DNase. The bacterial fusion proteins fixed on the nylon filter are now incubated with mAb1.1ASML and, after washing out of non-specifically-bound mAk, used for the detection of the binding as secondary antibody 125J-labelled rabbit anti-mouse IgG. After autoradiography, positive clones were isolated from the original bacteria filter and substantially analysed. One clone, which synthesised a fusion protein which reacted specifically with 1.1ASML, was pEX34. The pEX contained in the bacterial clone carries 167 nucleotide cDNA which, inter alia, codes for the epitope (or the antigen determinants), the specificity of which is carried by mAb1.1ASML.

The isolation of the total length cDNA mMeta-1 then took place according to standard methods.

Immunisation of the rats with mAb1.1ASML

BDX rats, which are syngenic to the BSp73 tumour cells, were injected subcutaneously or intraperitoneally with mAb1.1ASML (coupled to keyhole limpet haemocyananine), together with complete Freund's adjuvant. The first took place 10, 7 and 3 days before the injection of the BSpASML cells (into the fatty foot pad), the following then 3, 7, 11, 14 and 21 days thereafter. After 28 days, the rats were sacrificed, the various lymph nodes prepared and weighed and macroscopically visible lung metastases counted.

Connection between the expression of variant CD44 surface proteins and metastatic potential

In order to ascertain whether the expression of variant CD44 glucoproteins is merely a property of the investigated BSp73ASML cell line or whether the expression can be brought into connection with the metastatic potential, another series of rat tumour cells, which are derived from the 13762NF mammary carcinoma (Neri et al., 1982), were investigated. Furthermore, cell lines which were derived from the primary tumours (MTPa, MTC, MTF7 and MTA (group 1)) were compared with cell lines which are derived from lymph nodes and lung metastases (MTLy, MTLn2, MTLn3 (group 2)). The pattern of the RNA derived from CD44 is given in Figure 4, whereby samples A, B and D correspond to the samples described on pages 5 and 6 of the Application, as well as to Figure 1. Cells of group 1 all show a normal CD44 pattern with sample B. However, cells of group 2 show a pattern different therefrom. The RNA is larger than the RNA of group 1 and corresponds to the RNA of BSp73ASML. Smaller RNAs are lost in the case of the hybridisation with sample D.

The other patterns show the similarity between the two rat tumour systems.

Also with the sample A, the RNA pattern of group 2 corresponds to that of BSp73ASML. Whereas sample A does not hybridise with RNA from BSp73AS, there is shown a small diffuse RNA band of about 2.5 kb in the case of cells of group 1. RNase and S1 protection analysis also show the structural similarity. From these results, an exchange in the cleavage pattern and the expression of variant CD44 RNAs appears to take place with the formation of metastases.

Transfer of the metastatic potential to non-metastasing BSp73AS cells by overexpression of pMeta-1.

The connection of the expression of variant CD44 species with the metastatic potential in two series of rat tumours indicates a causal role of the glycoproteins in the metastatic process. In order to investigate this, pMeta-1 was transferred into BSp73AS cells and investigated whether the behaviour of the cells is thereby changed. The complete coding region of the pMeta-1 (Fig. 2) was inserted below the SV40 promotor and this formation (diagram in Figure 5) introduced into the BSp73AS cells, together with PSV2neo. Individual G418-resistant and pMeta-1-expressing colonies were obtained. The RNA pattern of 2 of these colonies is shown in Figure 5. The hybridisation of the variant CD44-specific sample A shows a dominant transcript of approximately 2.2 kb which corresponds to the size of the smallest frequent RNA which is transcribed in BSp73ASML cells (Figure 5). However, the transfected cells contain about 10 times as much of this RNA as BSp73ASML.

Other size orders are observed in one of the transfected cells (BSp73AS-pSVMeta-1-14), which could be dependent upon the place of the plasmid integration.

A pSV2neo simulation transfer clone (not shown) and the BSp73AS receiver cells contain no RNA which is complementary to sample A. In order to discover the endogenic normal CD44 transcriptions (without the extra domains of the pSVMeta-1) in the transfects, the filter was stripped and rehybridised with sample D. This part of the non-transferred 3' sequence is not contained in the expression clone (cf. Figure 5). Sample D detects two main transcripts of 2.9 and 4.9 kb in the RNA of the two transfects (Figure 5, right column), not only in the control BSp73AS but also in the non-illustrated BSp73ASpSVneo.

Approximate quantifications of the various agreeing hybridisations show that the transfects express approximately 5x as much of the variant CD44 RNAs, which are transcribed by the expression plasmid, as the endogenic gene transcripts.

The overexpressed cDNA is transferred into a protein. The two transfects, which are illustrated in Figure 5, synthesise mAb1-immune-colourable proteins of the same apparent size, namely, a main product of 150 kDa and a weaker band at 100 kDa. Since the cDNA sequence codes a primary protein product of only 503 amino acids (corresponds to about 60,000 Dalton), all visible bands must represent modified forms. The 150 kDa band runs together with one of the modified forms of variant CD44 which is expressed in the ~~metastasing~~ metastasising cells BSp73ASML. BSp73AS or simulation-transferred BSp73ASpSVneo do not possess this protein. As in BSp73ASML cells, the epitope of the cells expressed by the transfects lies freely on the cell surface.

In order to demonstrate that the expression of variant CD44 suffices in order to impart a metastatic potential to BSp73AS cells, transfects were injected

into syngenic BDX rats (spontaneous metastasis protocol). In earlier experiments, metastatic tumour cells BSp73ASML spread out quickly from the place of the injection and were completely distributed about 10 days after the injection (Matzku, 1984). All local tumours were, therefore, removed by amputations on the 10th day. All carriers of BSp73ASML cells and all animals which had been injected with an overexpressing transfect developed lung metastases (Table 1). The course of the metastasis formation was comparably quick within 5 - 8 weeks after the injection. Animals which had received Bsp73AS cells or simulation transfects were, after this time, completely healthy (apart from due to the amputation) and even after 5 months no metastases could be ascertained.

In spite of a surprising similarity in the strong metastasis formation, there are some interesting differences. In all animals, BSp73ASML cells reach the lymph nodes and lead to a massive enlargement of various nodes in the region of the inguinal groin and next to the aorta (Table 1). A transfect (BSp73AS-pSV meta-1-14) causes lymph node enlargement in 3 of 8 animals although all animals develop lung metastases (Table 1). No lymph node enlargement is ascertainable with the other transfect (BSp73AS-pSV Meta-1-15). The transfects appear, therefore, to be able to form colonies in the lungs without an obligatory growth phase of the lymph nodes.

The experiment according to Table 1 further points to another difference between BSp73AS transfects and BSp73ASML. The individual lung metastases are macroscopically visible, whereas those of BSp73ASML are small and numerous but, in a larger series with BSp73ASML (Reber et al., 1990), 11 of 20 animals develop 5 - 20 larger nodes per lung than the transfects.

In order to ascertain that the metastases formed were brought about by the injected transfects and in order to exclude the improbable possibility of a spontaneous mutation, which transfers a metastatic potential, the epitope-positive proteins in the total lung extracts and in the extracts of recultured metastasis-producing cells were determined. The 150 kDa glycoprotein is detectable in the whole lung extract, as well as in the extracts of a specific lung node from an animal which has received BSpAS-pSV meta-1-15 transfects. In the case of in vitro growth, the G418-resistant strain expresses a protein of the same apparent molecular weight.

Diagnosis and therapy

1. Analysis of human tumour material by in situ hybridisation with the human pMeta-1 sequence present. These experiments are considered as preliminary experiments before an Ab is available which recognises the human ECR.

2. Production of antibodies against the human ECR. Cloning of the human pMeta-1 sequences in bacterial expression vectors so that fusions arise with β-galactosidase or tryptophane E-product. Immunisation of rabbits with these fusion proteins or with synthesised peptides from the ECR (coupled to carrier molecules). Isolation of the polyvalent or monospecific antibodies.

Possibilities of use:

- Immunohistological investigations of clinical tumour material (diagnosis)
- Detection of soluble ECR in the serum of patients with the help of ELISA tests (diagnosis)
- Construction of toxin-coupled antibodies in order, with the help of the antibody, to bring the toxin into the tumour/metastasis region (therapy)

- Construction of antibodies with two definite antigen binding positions. By means of this double specificity, the attempt is to be made to initiate cytotoxic reactions in the ~~metastasis~~ metastatic region (e.g. anti CD2 or CD3 coupling) (therapy).

3. Production of hMeta-1 protein by transfection of human or rat cells with an expression vector which carries the complete hMeta-1 cDNA sequence; or purification from LCLC97 cells.

Possibilities of use:

- Injection of the protein or parts thereof in order to block the tissue binding positions of the tumour cells
- After characterisation of the binding positions, a use for therapy would also be conceivable which could depend upon the injection of large amounts of binding protein which would then block the migrating tumour cells.

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Table 1

Metastatic spreading out of BSp73AS cells which express variant CD44 cDNA pMeta-1***

tumour clone	local appear-ance	distribution in the case of metastatic autopsy		
		*LN ing	*LN par	lung
BSp73ASML	0/8	8/8 Ø 1.5-2.5**	8/8 Ø 2.5-5.0	8/8 miliary
BSp73AS-pSVMeta-1-14	0/8	3/8 Ø 0.3-1.2	3/8 Ø 1.0-4.5	8/8 multiple Ø 0.3-5.0
BSp73AS-pSVMeta-1-15	0/8	0/8	0/8	8/8 5-20 Ø 0.3-10.0
BSp73AS	1/8	0/8	0/8	0/8
BSp73AS-pSVneo	0/8	0/8	0/8	0/8

** average diameter in mm

*** the Table gives the stage 60 days after injection of the given cells

* LN = lymph nodes

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. DNA fragment, which codes for a part of a surface protein of a ~~metastasing~~ metastasising tumour cell, whereby this DNA fragment is selected from

a) the nucleotide sequences

...rMeta-1...

ATT GCA ACT ACT CCA TGG GTT TCT GCC CAC ACA AAA CAG AAC
CAG GAA CGG ACC CAG TGG AAC CCG ATC CAT TCA AAC CCA GAA
GTA CTA CTT CAG ACA ACC ACC AGG ATG ACT GAT ATA GAC AGA
AAC AGC ACC AGT GCT CAT GGA GAA AAC TGG ACC CAG GAA CCA
CAG CCT CCT TTC AAT AAC CAT GAG TAT CAG GAT GAA GAG GAG
ACC CCA CAT GCT ACA AGC ACA ACC TGG GCA GAT CCT AAT AGC
ACA ACA GAA GAA GCA GCT ACC CAG AAG GAG AAG TGG TTT GAG
AAT GAA TGG CAG GGG AAG AAC CCA CCC ACC CCA AGT GAA GAC
TCC CAT GTG ACA GAA GGG ACA ACT - GCC TCA GCC CAC AAC
AAC CAT CCA AGT CAA AGA AGT ACA ACA CAG AGT CAA GAG GAT
GTT TCA TGG ACC GAT TTC TTC GAC CCA ATC TCA CAT CCA ATG
GGA CAA

and

hMeta-1...

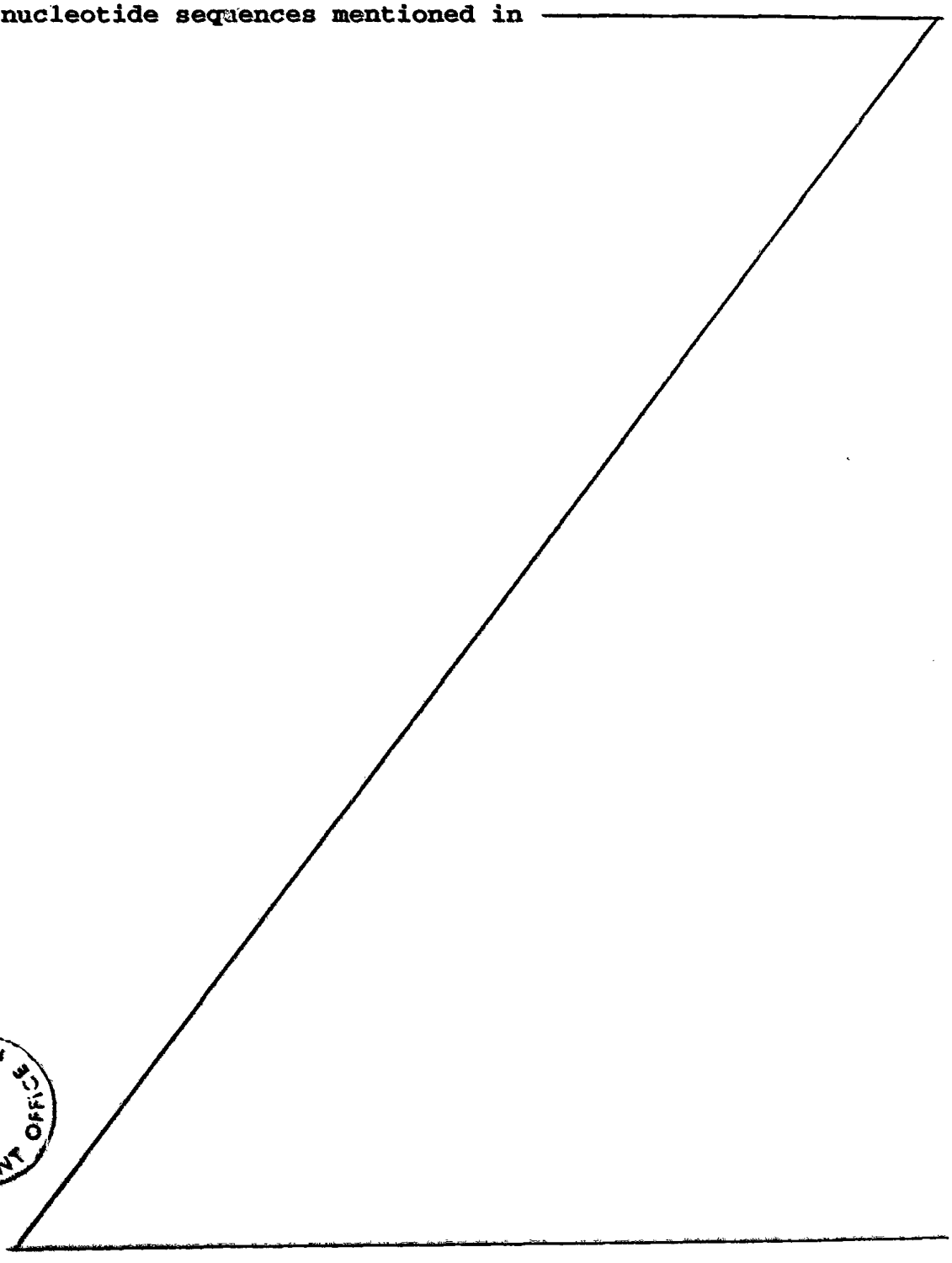
AAC CCA AGC CAT TCA AAT CCG GAA GTG CTA CTT CAG AGA ACC
ACA AGG ATG CAT GAT GTA GAC AGA AAT GGC ACC ACT GCT TAT
GAA GGA AAC TGG AAC CCA GAA GCA CAC CCT CCC CTC ATT CAC
CAT GAG CAT CAT GAG GAA GAA GAG ACC CCA CAT TCT ACA AGC
ACA ATC CAG GCA ACT CCT AGT AGT ACA ACG GAA GAA ACA GCT
ACC CAG AAG GAA CAG TGG TTT GGC AAC AGA TGG CAT GAG GGA
TAT CGC CAA ACA CCC AGA GAA GAC TCC CAT TCG ACA ACA GGG
ACA GCT GCA GCC TCA GCT CAT ACC AGC CAT CCA ATG CA

b) nucleotide sequences which encode the same peptide encoded by the DNA sequences mentioned under a) above;

c) nucleotide sequences which encode a surface protein representing allelic variations of the surface peptide encoded by the DNA sequences mentioned under a) above; or

d) any other nucleotide sequence which encodes a peptide with conservative amino acid changes, and having the biological function of the peptide encoded by the DNA sequences mentioned in a), b) or c) above.

2. DNA fragment according to claim 1, containing a nucleotide sequence which hybridises with one of the nucleotide sequences mentioned in



claim 1 a) and code for a complete surface glycoprotein of a ~~metastasising~~ metastasising tumour cell.

3. DNA fragment according to claim 2, characterised in that it is hybridised with one of the nucleotide sequences mentioned in claim 1 which displays at least 85% homology to the reaction partner.

4. Recombinant DNA molecule, consisting of a vectorial nucleotide sequence and a DNA fragment according to claims 1, 2 or 3.

5. Recombinant DNA molecule according to claim 4, characterised in that it is an expression vector.

6. Transformed host cell which contains a DNA fragment according to claims 1, 2 or 3 or a recombinant DNA molecule according to claims 4 or 5.

7. Polypeptide which is coded by a DNA fragment according to claim 1.

8. Polypeptide producable by a recombinant DNA molecule according to claims 4 or 5 which includes a DNA fragment according to claim 1.

9. Polypeptide according to claims 7 and 8, including the amino acid sequences

...r-protein...

I A T T P W V S A H T K Q N Q E R T Q W N P I H S N P E V L
L Q T T T R M T D I D R N S T S A H G E N W T Q E P Q P P F
N N H E Y Q D E E E T P H A T S T T W A D P N S T T E E A A
T Q K E K W F E N E W Q G K N P P T P S E D S H V T E G T T
A S A H N N H P S Q R M T T Q S Q E D V S W T D F F D P I S
H P M G Q G H Q T E S K

and h-protein

I S S T I S T T P R A F D H T K Q N Q D W T Q W N P S H S N
P E V L L Q T T T R M T D V D R N G T T A Y E G N W N P E A
H P P L I H H E H H E E E E T P H S T S T I Q A T P S S T T
E E T A T Q K E Q W F G N R W H E G Y R Q T P R E D S H S T
T G T A A A S A H T S H P M Q G R T T P S P E D S S W T D F
F N P I S H P M G R G H Q A G R R

and their allele or variations and glycosylation products.

10. Poly- or monovalent antibody which reacts with an epitope of a polypeptide according to claims 7 to 9.

11. Use of a DNA fragment according to claim 1, its complementary strand, derivatives and parts thereof for the identification, production or isolation of a nucleotide sequence or parts thereof which code for a part of a surface glycoprotein of a metastasising tumour cell.

12. Use of an antibody according to claim 10 for the identification, production or isolation of a poly-peptide according to claims 7 to 9.

13. Agent for the diagnosis of a metastasising tumour and/or metastasis which contains at least one antibody according to claim 10.

14. Agent according to claim 13, characterised in that the antibody is coupled with an enzyme which is labelled with a coloured material and/or with a radioisotope.

15. Antibody against proteins according to claim 7, produced with the use of DNA according to claim 1 or proteins according to claim 7.

16. Use of an antibody and protein or parts thereof according to claims 15 and 17 for the production of a medicament which is used in tumour therapy.

17. Use of an antibody according to claim 15 which is coupled with a cytotoxic agent for the production of a medicament for tumour therapy.

DATED this 3rd day of January 1994

KERNFORSCHUNGSZENTRUM KARLSRUHE GmbH
UNIVERSITAT KARLSRUHE and
DEUTSCHES KREBSFORSCHUNGSZENTRUM
By Its Patent Attorneys

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1/7

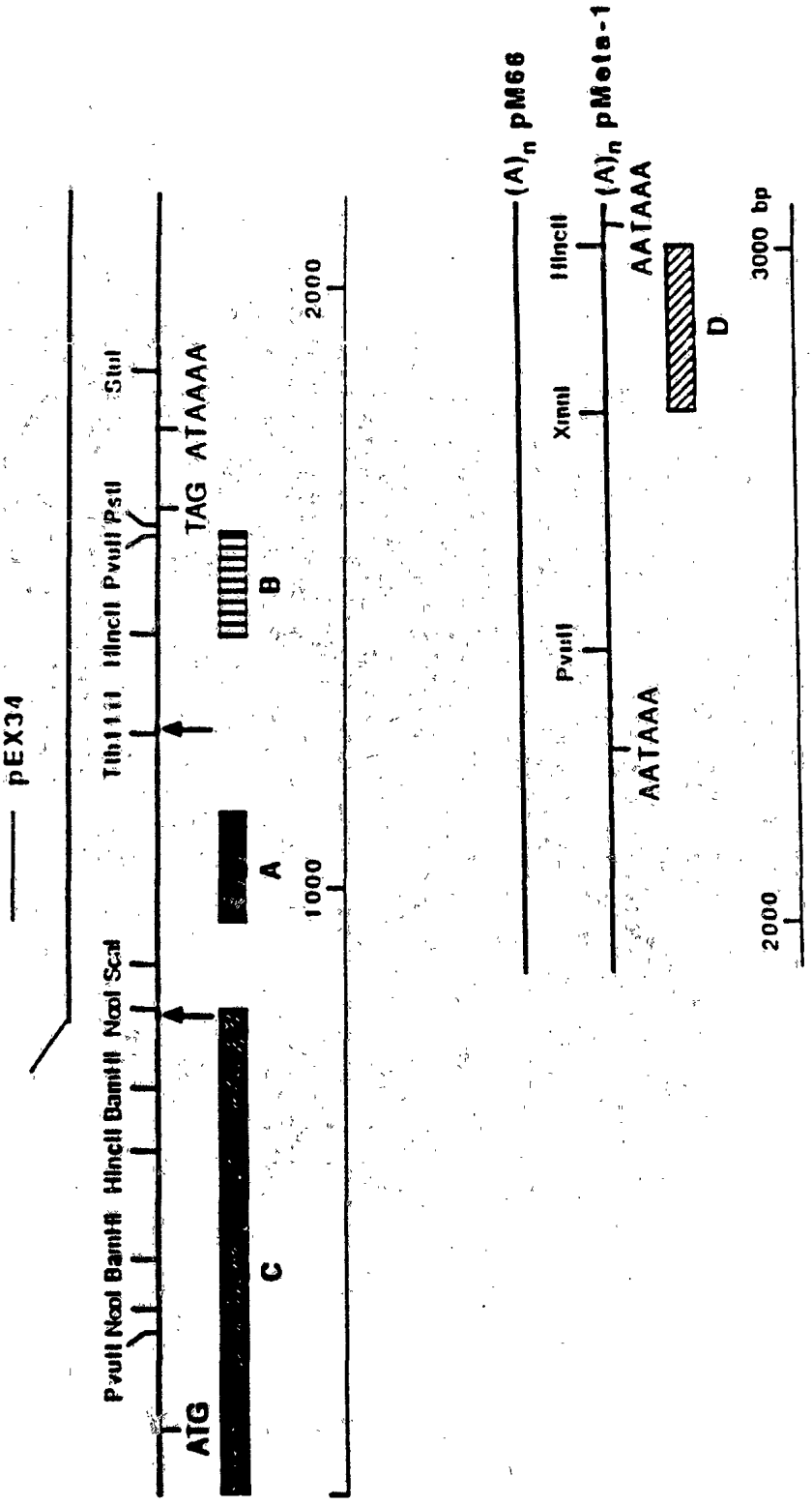


Abb. 1 Oberflächenprotein der Ratte p-Meta-1 mit Schnittstellen der Restriktionsenzyme und Antikörperhybridisierungsbereiche

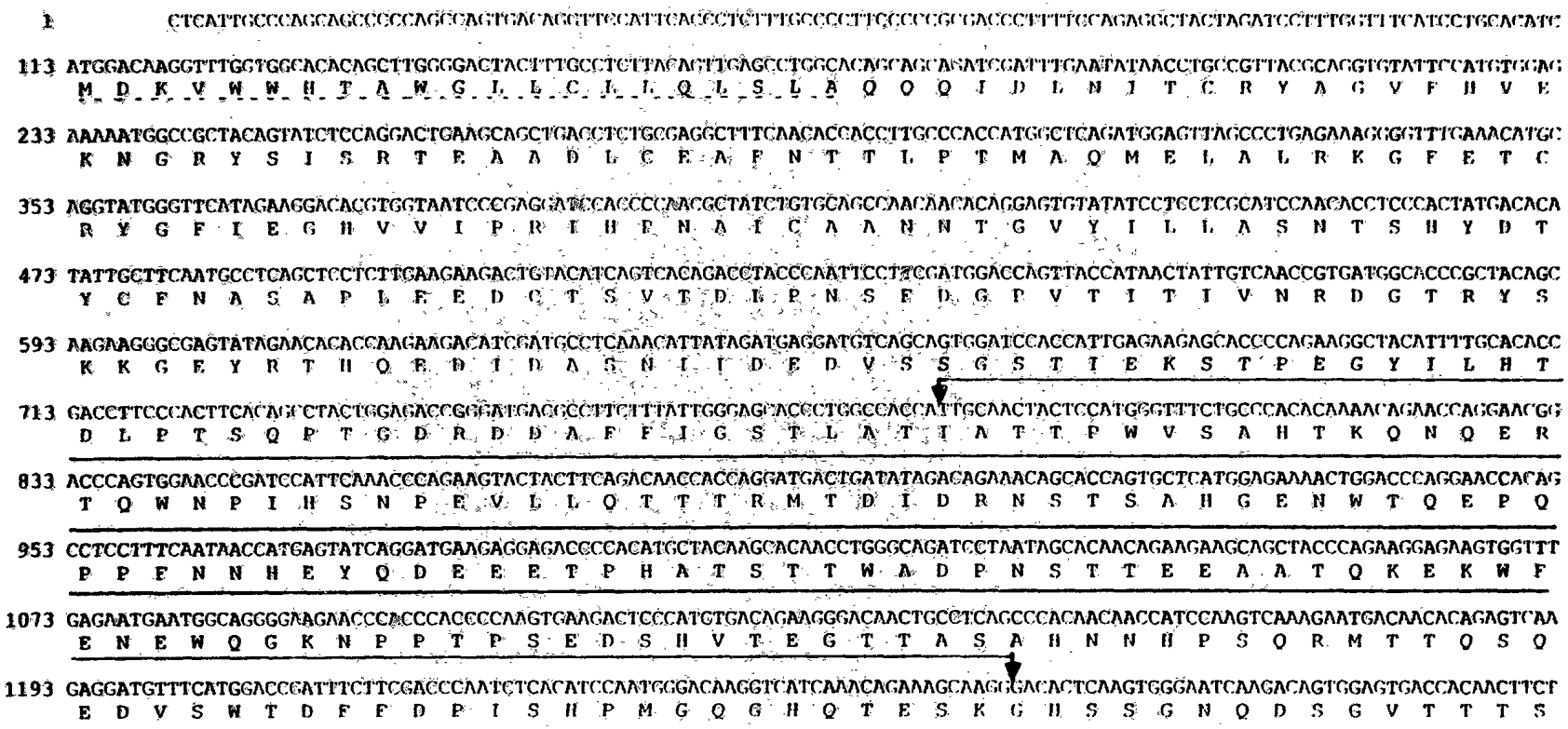


Abb. 2

Totalsequenz der DNA u. des CD44-Proteins von p-Meta-1 (Blatt 1)

.....Signalpeptid EZB Transmembranregion

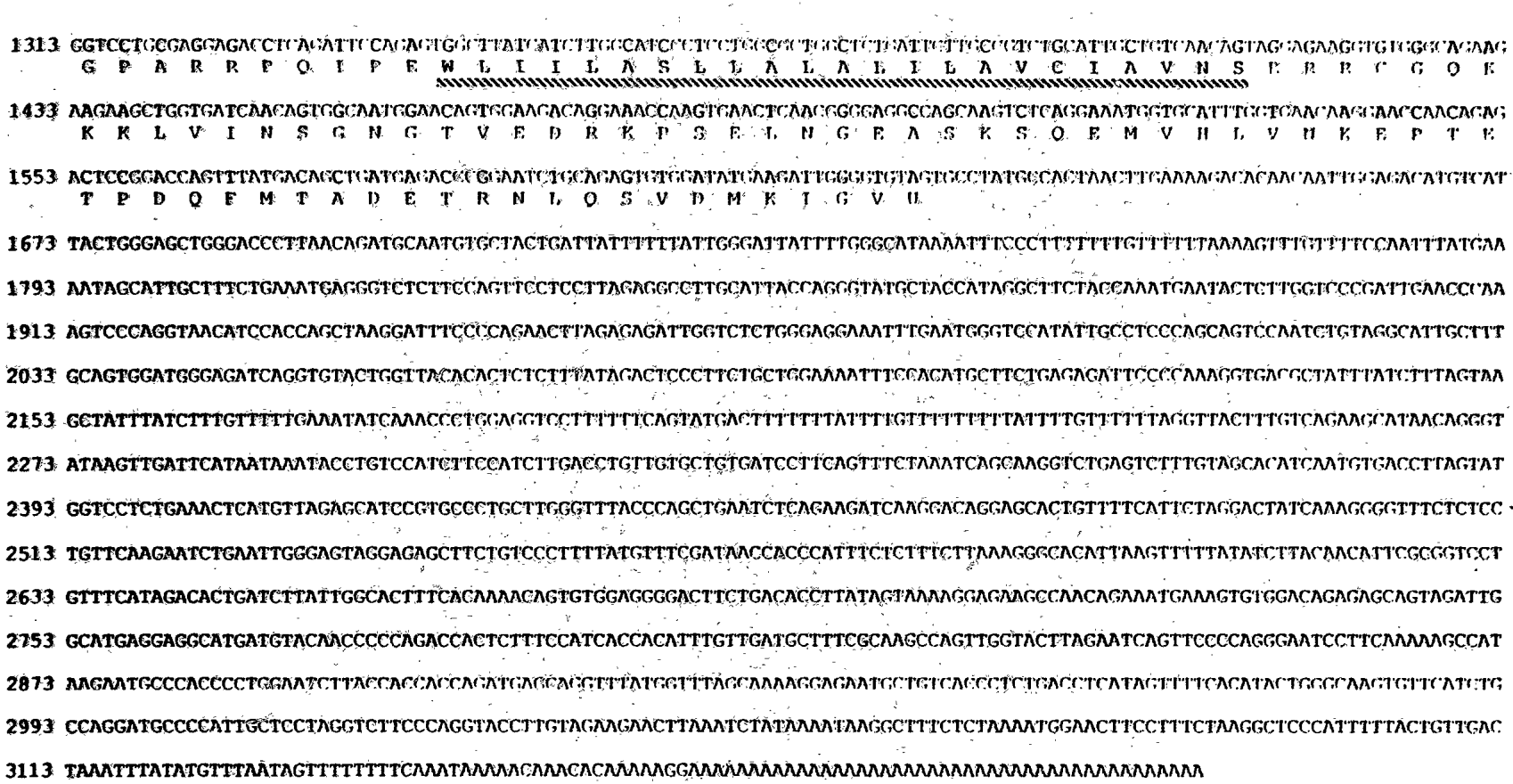
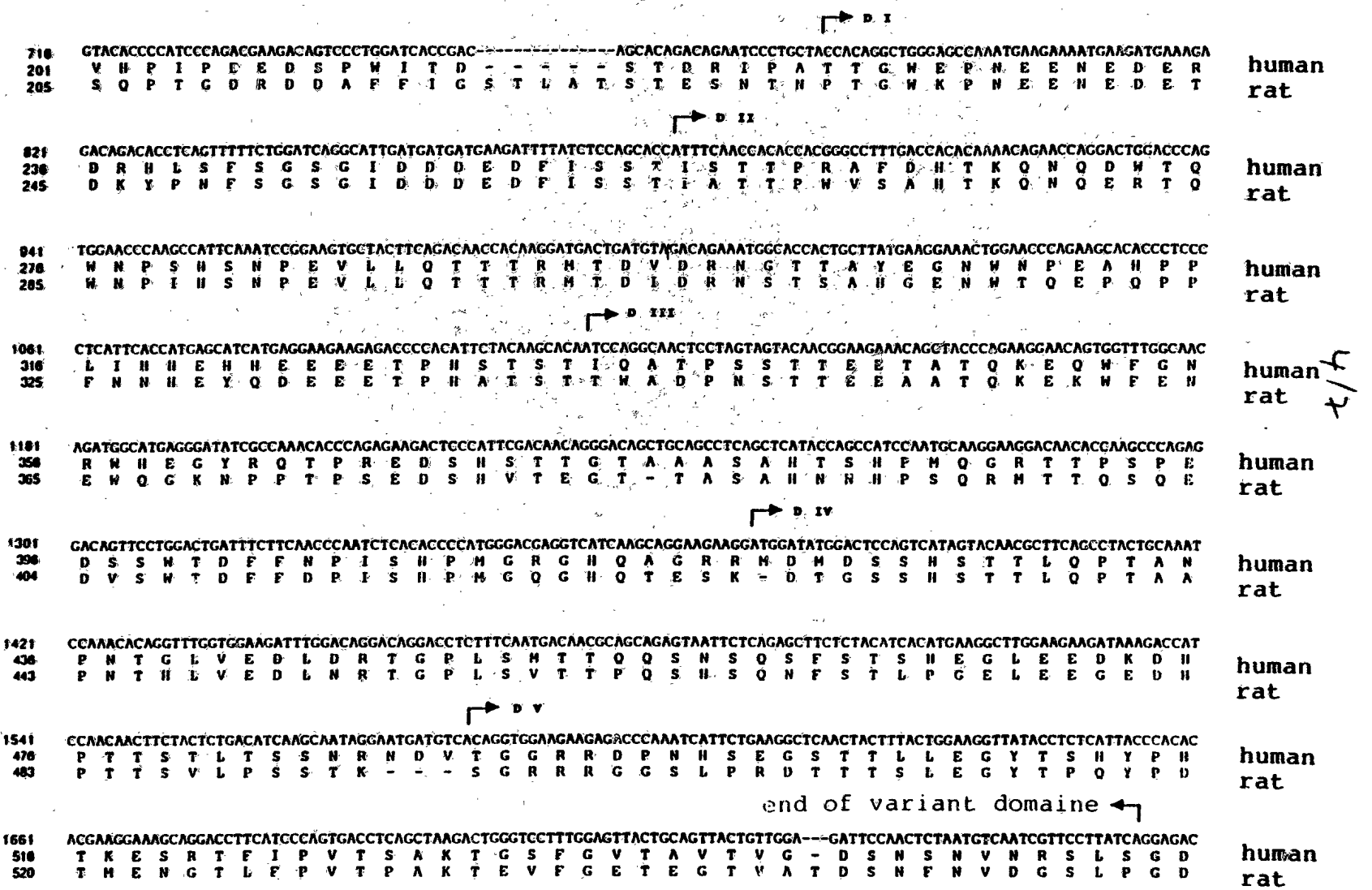


Abb. 2

Totalsequenz der DNA u. des CD44-Proteins von p-Meta-1 (Blatt 2)

Abb. 3a c-DNA und Proteinsequenz der extracellulären Bereiche



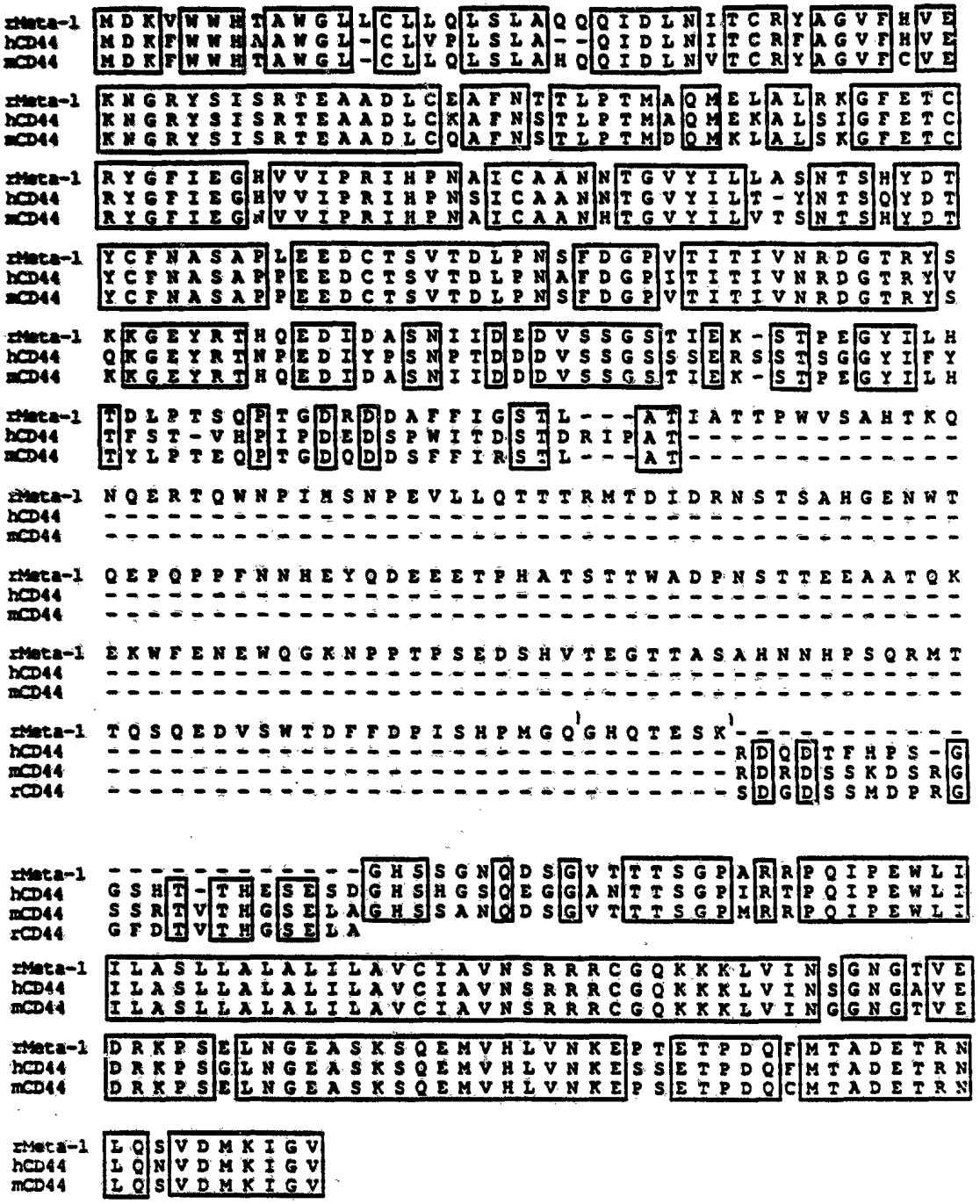
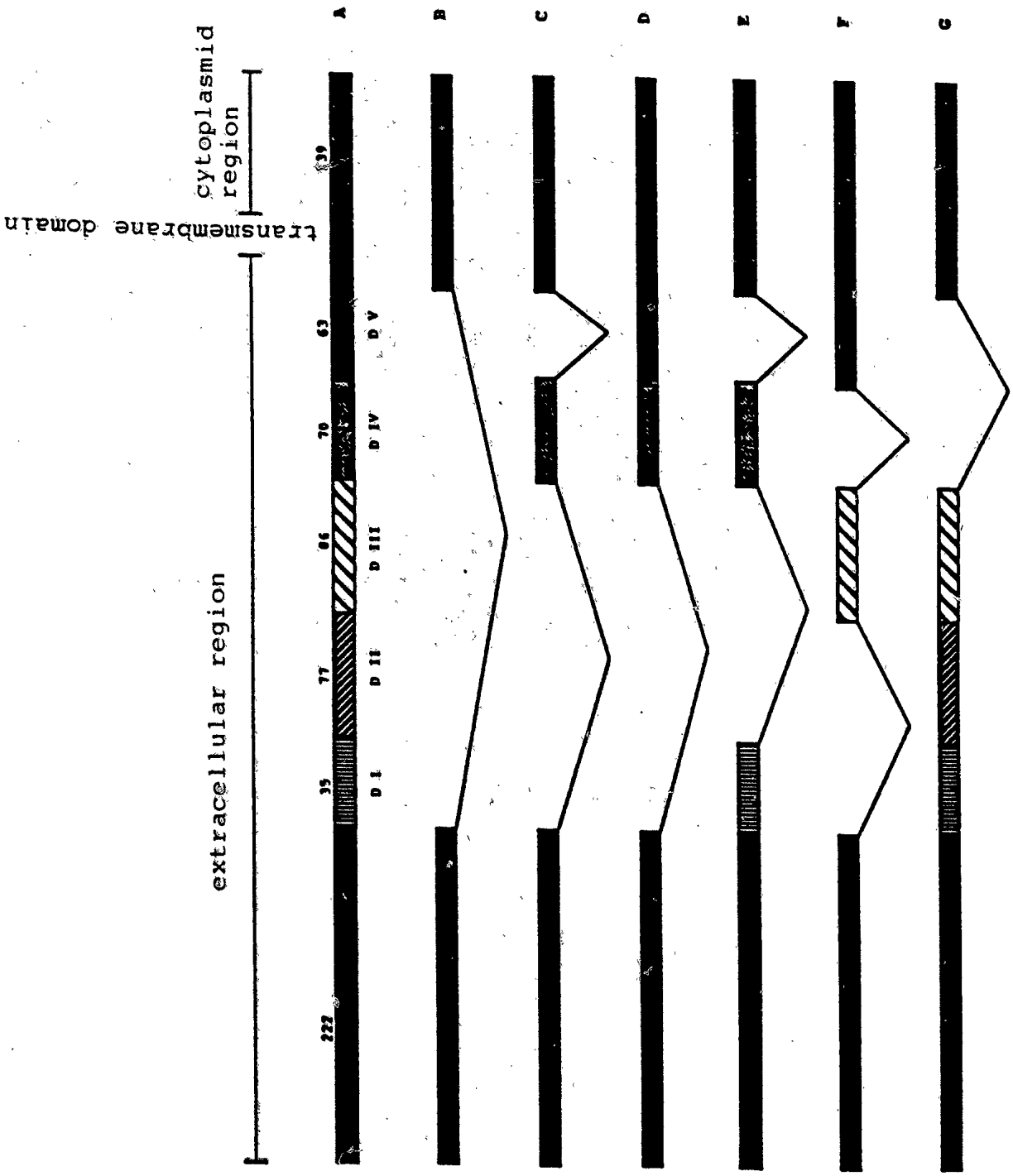


Abb. 3b

Proteinsequenz von Oberflächenproteinen von Ratten (rMeta-1) Mensch (hCD44) und Maus (mCD44) mit extracellulären Bereichen bei Ratten- und Humanprotein.

Fig. 4


A
B
C
D
E
F
G
cytoplasmid region
transmembrane domain
extracellular region
39
63
70
86
77
35
222
D V
D IV
D III
D II
D I

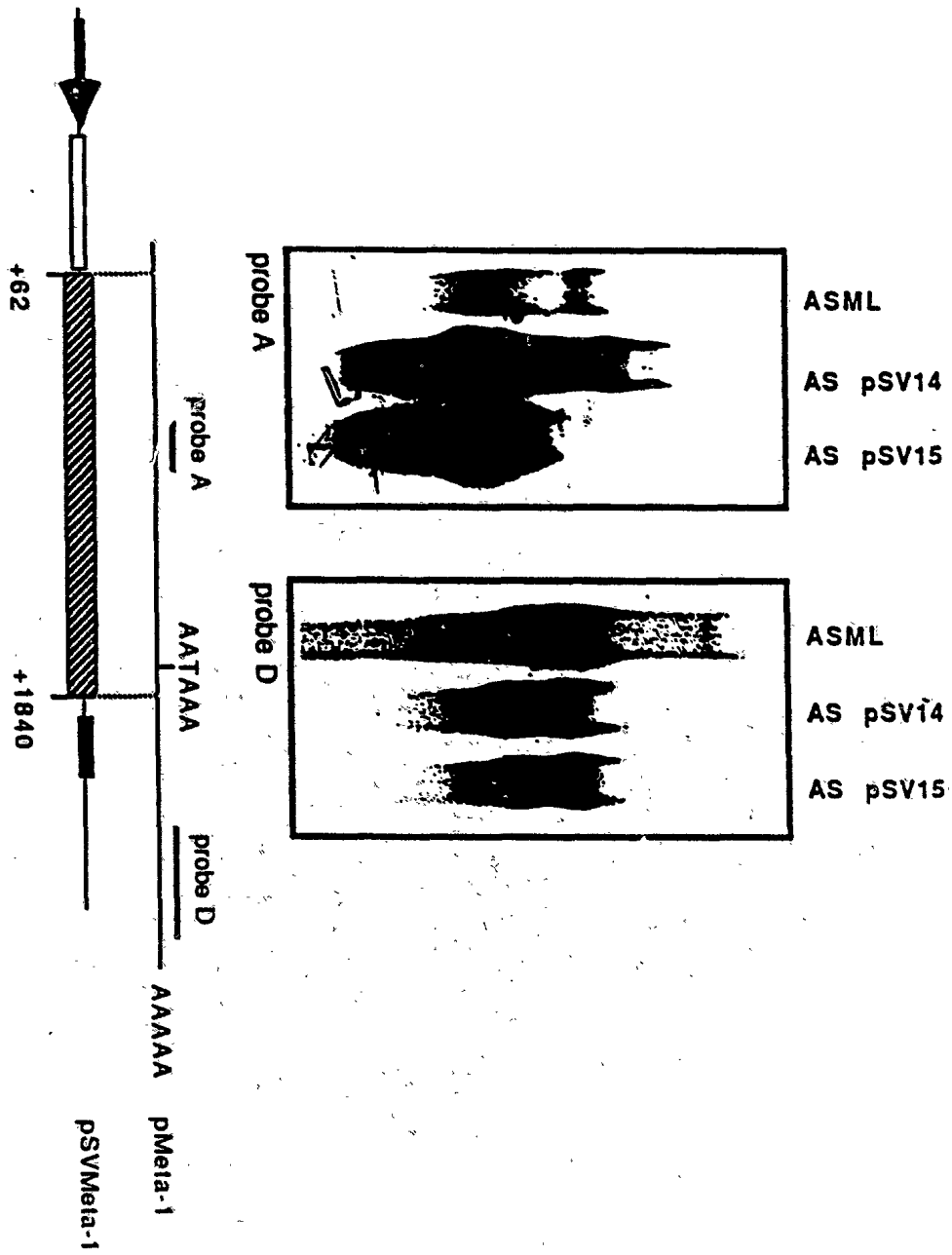

+62
+1840
probe A
AATAAA
probe D
AAAAA
pMeta-1
pSVMeta-1
probe A
ASML
AS pSV14
AS pSV15
probe D
ASML
AS pSV14
AS pSV15

Fig. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 91/00614

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.[5]: C 12 N 15/12, C 12 P 21/08, A 61 K 37/02, C 07 K 13/00, G 01 N 33/514

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
Int.Cl.[5]:	C 12 N, C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Cancer Research, vol. 49, 1989, S. Matzku et al.: "Antigenic differences between metastatic and nonmetastatic BSp73 rat tumor variants characterized by monoclonal antibodies", pages 1294-1299 see abstract; page 1294, column 2, examples 5,6; page 1295, column 1, example 1; "discussion" cited in the application	9,11-14,16
A	Anticancer Research, vol. 8, No: 6, December 1988, M. Birnbaum et al.: "Amplification, expression and localization of the c-myc gene in Bsp73 rat tumor cell lines", pages 1185-1192 see the whole document cited in the application	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12 July 1991 (12.07.91)	30 August 1991 (30.08.91)

International Searching Authority	Signature of Authorized Officer
European Patent Office	

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen PCT/EP 91/00614

I. KLASSIFIKATION DES ANMELDUNGSGEGENSTANDS (bei mehreren Klassifikationssymbolen sind alle anzugeben)[6]

Nach der Internationalen Patentklassifikation (IPC) oder nach der nationalen Klassifikation und der IPC

Int.Cl.[5] C 12 N 15/12, C 12 P 21/08, A 61 K 37/02, C 07 K 13/00, G 01 N 33/514

II. RECHERCHIERTE SACHGEBIETE

Recherchierter Mindestprüfstoff[7]

Klassifikationssystem	Klassifikationssymbole
Int.Cl.[5]	C 12 N, C 07 K

Recherchierte nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Sachgebiete fallen[8]

III. EINSCHLÄGIGE VERÖFFENTLICHUNGEN[9]

Art*	Kennzeichnung der Veröffentlichung[11], soweit erforderlich unter Angabe der maßgeblichen Teile[12]	Betr. Anspruch Nr.[13]
X	Cancer Research, volume 49, 1989, S. Matzku et al.: "Antigenic differences between metastatic and nonmetastatic BSp73 rat tumor variants characterized by monoclonal antibodies", Seiten 1294-1299 siehe Zusammenfassung; Seite 1294, Spalte 2, Beispiele 5,6; Seite 1295, Spalte 1, Beispiel 1; "discussion" in der Anmeldung erwähnt	9,11-14,16
	--	
A	Anticancer Research, Band 8, Nr. 6, Dezember 1988, M. Birnbaum et al.: "Amplification, expression and localization of the c-myc gene in Bsp73 rat tumor cell lines", Seiten 1185-1192 siehe das ganze Dokument in der Anmeldung erwähnt	
	---	

* Besondere Kategorien von angegebenen Veröffentlichungen[10]:

"A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist

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"O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht

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"Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist

"&" Veröffentlichung, die Mitglied derselben Patentfamilie ist

IV. BESCHEINIGUNG

Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
12. Juli 1991	30. 08. 91
Internationale Recherchenbehörde	**Unterschrift des bevollmächtigten Bediensteten**
Europäisches Patentamt	M. PEIS ! M. Peis