Involvement of a nuclear matrix association region in the regulation of the SPRR2A keratinocyte terminal differentiation marker

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ABSTRACT
The small proline-rich protein genes (SPRRs) code for precursors of the cornified cell envelope, and are specifically expressed during keratinocyte terminal differentiation. The single intron of SPRR2A enhanced the activity of the SPRR2A promoter in transient transfection assays. This enhancement was position dependent, and did not function in combination with a heterologous promoter, indicating that the intron does not contain a classical enhancer, and that the enhancement was not due to the splicing reaction per se. Mild DNAse-I digestion of nuclei showed the SPRR genes to be tightly associated with the nuclear matrix, in contrast to the other cornified envelope precursor genes mapping to the same chromosomal location (epidermal differentiation complex). In vitro binding studies indicated that both the proximal promoter and the intron of SPRR2A are required for optimal association of this gene with nuclear matrices. Neither nuclear matrix association nor the relative transcriptional enhancement by the intron changed during keratinocyte differentiation. Apparently, the association of the SPRR2A gene with the nuclear matrix results in a general, differentiation-independent enhancement of gene expression.

INTRODUCTION
The epidermal differentiation complex (EDC) on human chromosome 1 band q21 comprises at least 30 genes clustered in a 2 Mb region (1,2). Three classes of protein are encoded by the genes identified thus far: the S100 calcium binding proteins, the intermediate filament-associated proteins filaggrin, trichohyalin and repentin, and the cornified envelope (CE) precursors loricrin, involucrin and small proline-rich proteins (SPRRs) (3,4). Currently, 13 S100 genes (5–7) and 10 SPRR genes (4) have been identified at 1q21. Structural homology between the different genes of one class (3,8,9), and the finding that the intermediate filament-associated proteins show characteristics of both the S100 proteins and the CE precursors (10–12), indicate that the whole gene cluster has coordinately evolved. Besides a related structure, the genes in the EDC also have a similar expression pattern. The expression of the CE precursors and intermediate filament-associated proteins is restricted to squamous epithelia (10,12–14); also, most of the S100 genes in the EDC are expressed in epidermal or mucosal epithelia (15–19). Transcriptional regulation of these genes thus appears to occur at two levels: a coordinated expression in squamous epithelia and individual regulation, which results in tissue specificity (e.g. epidermal or mucosal epithelium) or differential responsiveness to environmental signals (4,14,20,21). The molecular processes for these two mechanisms are poorly understood, but several cis-elements have been identified in the promoter regions of various genes in the EDC, revealing both common, and gene-specific elements (20,22–30).

We have previously analyzed the promoters of the SPRRIA (30) and SPRR2A genes (20) and have identified several regulatory elements required for expression during keratinocyte terminal differentiation. One common element is an Ets binding site, bound by the epitheliom-specific transcription factor ESE-1 (20,30,31). A binding site for ESE-1 has recently also been identified in the profilaggrin gene (32), indicating that this transcription factor might be involved in the coordinated epithelial expression of the genes in the EDC. A functional AP-1 binding site is found in SPRRIA, while an octamer binding site, bound by Oct-11, and an interferon-stimulated response element (ISRE) bound by IRF-1 and IRF-2, are specific for SPRR2A (20,30), suggesting that these transcription factors are among the factors involved in the individual gene regulation.

Transcriptional regulatory elements can be found in positions downstream of the transcription start site and can contribute significantly to gene expression (reviewed in 33); for example,
Terminal differentiation was induced by allowing stratification of experiments described here, passage number 3 was used. For foreskin derived from circumcision and grown in the presence of complete medium (20). Keratinocytes were isolated from primary cultures of human epidermal keratinocytes were initiated by calcium (1.8 mM) by a Fluor Diffusion CAT assay (36) using Econofluor II premixed scintillation fluid (NEN Research Products). Activity was measured 36 to 48 h after induction of stratification by calcium (1.8 mM) by a Fluor Diffusion CAT assay (36) using Econofluor II premixed scintillation fluid (NEN Research Products).

**DNA manipulations and sequence of the SPRR2A intron**

Subclones of λspr2A (37) spanning the SPRR2A intron were sequenced on both strands. pSG-2 is the 1500 bp SPRR2A promoter–CAT construct described in (37) which lacks the natural Asp718I restriction site at position +14; pSG-90 is the same construct, yet does contain this Asp718I site, and was used to create pSG-122 and pSG-136. The promoter activity of pSG-90 and pSG-2 is identical (D.F.Fischer, G.S.Winkler and C.Backendorf, unpublished observations). By site directed mutagenesis (GGACTC to GGTACC) an SPRR2A intron restriction site was introduced between the 3′ splice site and the translation initiation site on the second exon of SPRR2A (Fig. 1).

**Cell culture**

Primary cultures of human epidermal keratinocytes were initiated in complete medium (20). Keratinocytes were isolated from foreskin derived from circumcision and grown in the presence of 1.8 mM CaCl2 (20). HeLa cells and primary human fibroblasts (isolated from foreskin) were cultured in DMEM (Gibco BRL, Paisley, Scotland) with 10% newborn calf serum (Hyclone, Logan, UT) and primary human fibroblasts (isolated from foreskin) were cultured in DMEM (Gibco BRL, Paisley, Scotland) with 10% newborn calf serum (HyClone, Logan, UT).

**Transient transfections and CAT assay**

Transient transfections were performed according to (20). CAT activity was measured 36 to 48 h after induction of stratification by calcium (1.8 mM) by a Fluor Diffusion CAT assay (36) using Econofluor II premixed scintillation fluid (NEN Research Products, Boston, MA) and [3H]acetyl coenzyme A (Amersham Pharmacia Biotech, UK) according to the procedure proposed by NEN Research Products.

**Isolation and analysis of DNA from chromatin and nuclear matrix fractions**

Tissue culture cells were extracted to yield nuclear matrix and chromatin fractions according to a published protocol (39,40). Briefly, cells were lysed in cytoskeleton buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES–NaOH pH 6.8, 3 mM MgCl2, 1 mM EDTA, 1.2 mM phenylmethylsulfon fluoride, 2 mM vanadyl ribonucleoside) with 0.5% Triton X-100; skeletal frameworks were extracted once with 0.25 M ammonium sulfate. The chromatin fraction was isolated by DNase-I treatment at 20°C (100 µg/ml; Boehringer Mannheim) in the above-mentioned buffer with 50 mM NaCl, which was terminated by addition of ammonium sulfate to 0.25 M. Approximately 50% of the DNA, was solubilized by DNase-I during this procedure. Subsequently, both the nuclear matrix pellet and solubilized chromatin supernatant were treated with 200 µg/ml proteinase K (Boehringer Mannheim) for 6 h at 55°C in 40 mM Tris–Cl pH 8.0, 100 mM sodium acetate pH 8.0, 1 mM EDTA, 0.1% SDS. After phenol extraction and ethanol precipitation, 2.5 µg of DNA was loaded on a slotblot (Hybond-N+, Amersham, UK) and hybridized with gene-specific probes (41,42).

**Nuclear matrix association experiments**

Nuclei from rat liver cells were isolated as described by Izuarralde et al. (43) and were kept at –80°C in storage buffer [7.5 mM Tris–HCl pH 7.4, 40 mM KCl, 1 mM EDTA, 0.25 mM spermidine, 0.1 mM spermine, 1% (v/v) thiglycol, 0.2 M sucrose, 50% (v/v) glycerol] at a density of 102 nuclei/ml. Nuclear matrix preparation was essentially as described by Mirkovitch et al. (44). To obtain matrices for the binding assay, rat liver nuclei were subjected to the lithium 3,5-diiodosalicylate (LIS)-extraction protocol (45). Nuclei of 107 cells were rinsed once in 10 ml of washing buffer [3.73 mM Tris–HCl pH 7.4, 20 mM KCl, 0.5 mM EDTA, 0.125 mM spermidine, 0.05 mM spermine, 1% (v/v) thiglycol, 0.1% (w/v) digitonin and 20 µg/ml aprotinin]. After pelleting (300 g for 10 min at 4°C), the nuclei were carefully resuspended in 0.5 ml washing buffer and heat stabilized by incubation for 20 min at 42°C. Non-matrix proteins were removed by adding 10 ml of 10 mM LIS in extraction buffer.
obtained by subsequent restriction of the genomic DNA from the dIII and Hin standard binding assays, nuclear matrices from 10^6 cell equivalents (aprotinin). For the aprotinin and incubating for 15 min at 25°C, MgCl2, 0.125 mM spermidine, 0.05 mM spermine and 10 µg/ml aprotinin). For the in vitro assay nuclear matrices were obtained by subsequent restriction of the genomic DNA from the halos in 1 ml of digestion buffer with 1000 U each of EcoRI, HindIII and XhoI for 2 h at 37°C. The matrix preparations were subsequently adjusted to a final concentration of 15 mM EDTA and 80 µg/ml Escherichia coli genomic competitor DNA. For standard binding assays, nuclear matrices from 10^6 cell equivalents were incubated overnight at 37°C with 40 ng of the appropriate [α–32P]dATP end-labelled restriction fragments. After separation into pellet and supernatant fractions by centrifugation (15 000 g for 30 min at 4°C), DNA was purified by incubation at 37°C for 60 min with 0.1% SDS and 50 µg/ml proteinase K, followed by phenol–chloroform extraction. DNA was precipitated, dissolved in 50 µl 10 mM Tris–HCl pH 7.5, 1 mM EDTA, and subsequently half of the pellet, supernatant or input fractions were loaded on a 1.2% agarose–TBE gel which, after electrophoresis, was dried on Whatman 3MM paper. Autoradiography was overnight by exposure to Kodak X-OMAT S films.

RESULTS

The SPRR2A intron stimulates transcription of the SPRR2A gene in transient transfection

We have shown previously that the minimal promoter region, required for the differentiation-dependent expression of SPRR2A, maps to position −134 to +14 (Fig. 1) (20). This analysis was performed with constructs lacking the intron. When the intron is present at its natural position in a construct containing the full-length SPRR2A promoter, expression in transient transfections to primary human keratinocytes was enhanced 3– to 4-fold (Fig. 2, compare pSG-2 with pSG-122). Identical results were obtained with plasmids containing the minimal promoter region (compare pSG-55 with pSG-136). The displacement of the intron from its natural position to position −1500 abrogated the stimulatory effect (compare pSG-160 and pSG-161 with pSG-122). Furthermore, enhancement by the SPRR2A intron was specific for the SPRR2A promoter since the intron did not affect expression of the RSV promoter when inserted between this promoter and the CAT gene (compare RSV-CAT with pSG-258). These findings indicate that (i) the increase in expression is not due to RNA splicing per se (as a matter of fact, all constructs contain the SV40 small t intron downstream of the CAT gene); (ii) the intron does not contain a classical enhancer, which functions in a position- and orientation-independent manner (46,47) and (iii) the SPRR2A intron specifically enhances transcription of the SPRR2A promoter.

Similar experiments were performed with the SPRR1A gene. We have shown previously that a fragment of the SPRR1A gene from position −173 to +1147 (pSG-227) is sufficient for induction of this gene during keratinocyte terminal differentiation (38). Subsequent experiments indicated that the upstream fragment can be deleted up to position −125 without changing the promoter activity (Fig. 2, compare pSG-227 and pSG-425); interestingly, in the case of SPRR1A, the intron present in pSG-227 did not influence the level of expression (Fig. 2, compare pSG-227 and pSG-380). Sequence comparison of the SPRR1A intron with the SPRR2A intron by the GAP alignment program (Genetics Computer Group 9.1) showed no significant homology (40.4% identity over 1087 nt with an alignment score of 2465, whereas the score for the randomized sequence is 2441 ± 39). Apparently, the specific enhancer function in the intron of SPRR2A has been acquired after the divergence of these two genes. In contrast, the proximal promoter regions show still considerable homology (the region between the Ets binding site and the TA TA box is conserved for 57.7% over 45 nt).

Cornified envelope precursor genes are differentially located in accessible chromatin and at the nuclear matrix

Simple sequence inspection of the SPRR2A intron did not reveal consensus binding sites for transcription factors (data not shown), which prompted us to examine the SPRR2A gene for the presence of elements of higher order chromatin structure. Many gene complexes that are regulated in a concerted manner have been shown to contain DNase-I hypersensitive sites [e.g. the β-globin locus (48)] or nuclear matrix associated regions [MAR, e.g. the immunoglobulin κ gene (49)]. These latter elements are also referred to as scaffold attached regions (SAR) and are biochemically defined as DNA fragments that bind specifically to the nuclear matrix (reviewed in 35). Both DNase-I hypersensitive sites and MARs have been shown to affect transcriptional regulation (50–55). Interestingly, the intron of the S100A4 gene, also present in the EDC, was shown to contain both regulatory elements and DNase-I hypersensitive sites (56). We have examined several 5–8 kb restriction fragments from the SPRR gene cluster for the presence of DNase-I hypersensitive sites, but did not observe these (data not shown). In contrast, the SPRR2 genes were strongly protected from mild DNase-I treatment and remained bound to the nuclear matrix fraction, whereas the more accessible DNA was found in the soluble fraction (39,40).
In this study we have identified a nuclear MAR which localizes to the promoter and intron of the SPRR2A gene. The data

Comparison of the binding affinity of various DNA fragments to rat liver nuclear matrices in vitro. The ratio between DNA bound to the nuclear matrix and soluble DNA remaining in the supernatant was determined by scanning of autoradiograms and evaluation with the NIH Image program version 1.61. The average results of two experiments are presented.

These observations prompted us to examine the enhancing activity of the intron at different stages of terminal differentiation. The SPRR2A intron apparently does not confer terminal differentiation responsiveness as the relative contribution to transcription did not change during keratinocyte terminal differentiation (Fig. 5A, compare 12 h with later times). Activity of this enhancer could neither be measured in undifferentiated keratinocytes (Fig. 5B; 0 and 6 h), nor in primary human fibroblasts (data not shown), since CAT activity of either construct was identical to the activity of vector DNA (Fig. 5B, triangle), which suggests that the enhancing activity of the intron requires the activity of the promoter, which is clearly dependent on the differentiation state of the transfected cell (20). Furthermore, the activity in differentiated keratinocytes of the SPRR2A construct with intron (pSG-122 and pSG-136) was still dependent on the presence of each of the four terminal differentiation elements identified previously in the SPRR2A promoter (20) (data not shown).

**DISCUSSION**

Localisation of the matrix association region in the SPRR2A gene

In contrast to the more accessible SPRR1 and SPRR3 genes, the SPRR2 genes were tightly associated with the nuclear matrix. The nature of this association was further examined in in vitro binding experiments. Several fragments of the SPRR2A and SPRR1A genes were tested for their ability to associate with rat liver nuclear matrices in the presence of E.coli genomic DNA fragments as competitor. Fragments containing the SPRR2A promoter (HindIII–Asp718I, position –1500 to +14) or the SPRR2A intron (Asp718I–Asp718I, +14 to +766, Fig. 1) bound strongly to isolated nuclear matrices (Fig. 4B, lane 2). Interestingly, a fragment containing the promoter, the untranslated first exon of 42 bp and the intron (HindIII–HindIII, –1500 to +697) had a higher affinity than each of the separate fragments (Fig. 4A, lane 2; Table 1). For efficient binding to nuclear matrices, a fragment containing the minimal promoter region associated with the intron was sufficient (Fig. 4A, lane 5). The SPRR1A promoter and intron did not associate with nuclear matrices in vitro, neither when tested separately nor as one DNA fragment (Fig. 4C, lanes 2 and 5, respectively). These data corroborated the results obtained by mild DNAse-I treatment of isolated nuclei, which indicated that SPRR2 was bound to the nuclear matrix in vivo. The characteristics of the nuclear matrix association of SPRR2A are thus: (i) association is independent of the cellular differentiation status; (ii) both the intron and the promoter regions contribute to the overall binding affinity.

**Table 1. Association of SPRR2A DNA fragments with rat liver nuclear matrices**

<table>
<thead>
<tr>
<th>DNA fragment</th>
<th>Restriction sites</th>
<th>Ratio pellet to supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector DNA</td>
<td>HindIII + Asp718I</td>
<td>0.36 ± 0.16</td>
</tr>
<tr>
<td>1500 bp promoter</td>
<td>HindIII + Asp718I</td>
<td>1.17 ± 0.04</td>
</tr>
<tr>
<td>Intron</td>
<td>Asp718I</td>
<td>1.09 ± 0.19</td>
</tr>
<tr>
<td>1500 bp promoter + intron</td>
<td>HindIII</td>
<td>1.95 ± 0.64</td>
</tr>
</tbody>
</table>

Comparison of the binding affinity of various DNA fragments to rat liver nuclear matrices in vitro. The ratio between DNA bound to the nuclear matrix and soluble DNA remaining in the supernatant was determined by scanning of autoradiograms and evaluation with the NIH Image program version 1.61. The average results of two experiments are presented.
This rules out that the observed enhancement is due to RNA binding studies. In contrast, the in vitro localization of the presented strongly suggest that the MAR contributes to the promoter activity, for the following reasons: (i) the enhancement of gene expression by insertion of the intron was specific for the promoter region and not found for the RSV promoter. This rules out the observed enhancement is due to RNA splicing per se or another post-transcriptional event. (ii) The transcriptional enhancement can only be observed when the intron is present at its natural position; displacement of the intron to position −1500 eliminates the enhancing effects of the intron. This suggests that the intron does not contain a classical enhancer, which can influence gene expression in either orientation at many positions, as is the case for the prototypic SV40 enhancer (46,47). (iii) The intron of SPRR2A induced promoter activity whereas the intron of SPRR1A did not. This correlates with the in vivo localization of the SPRR2A gene at the nuclear matrix and with in vitro binding studies. In contrast, the SPRR1A gene neither bound to nuclear matrices in vitro, nor was protected from mild DNAse-I treatment of nuclei. (iv) The transcriptional enhancement by the SPRR2A intron was observed with both the full-length promoter and the minimal promoter region that is sufficient for SPRR2A expression after induction of terminal differentiation (20). In in vitro studies the region of the SPRR2A gene involved in the nuclear matrix association has been mapped to the minimal promoter region and the single intron. Both these regions cooperate for maximal binding to isolated nuclear matrices. This cooperativity between minimal promoter and intron is likely to be the molecular mechanism of the observed transcriptional enhancement (reviewed in 61). (v) The observed stimulatory effect (3- to 4-fold) of the intron on transcription was constant during progressive stages of keratinocyte differentiation, and was solely dependent on the activity of the SPRR2A promoter, which contains four terminal differentiation elements (20). Similarly, the association of SPRR2A with the nuclear matrix was constant during keratinocyte terminal differentiation.

Although the above-mentioned argumentation strongly suggests that the association of SPRR2A with the nuclear matrix has a positive effect on the expression of this gene, mutational analysis would be required to confirm this link. Such an analysis is, however, impeded by the fact that no clear consensus sequence for a MAR has been established (59,62). A major class of MARs has been defined as being A/T-rich and requiring a length of at least 160 bp for matrix association (35,59). The SPRR2A intron, which is moderately A/T-rich, would conform to these criteria. We have deleted an A/T-rich fragment of the SPRR2A intron (position +123 to +360, 68% A/T), but the remaining sequence still bound to nuclear matrices and still enhanced transcription of the SPRR2A promoter (data not shown). This indicates either that the deleted sequence is not involved in nuclear matrix association and transcriptional enhancement, or that the remaining sequences, which are also A/T-rich, can compensate for the deleted fragment. A more thorough mutational analysis of the intron is required to identify the regions important for matrix association and transcriptional activation. This is not a straightforward analysis, as these mutants should still allow efficient splicing. For the same reason, we have not reversed the orientation of the intron, as we have done for the same sequence when positioned at a distal location (pSG-160 and pSG-161). On the other hand, the SPRR2A MAR might not be a classical MAR (as defined above), as it is clearly composed of two adjacent fragments, one covering the intron and the other one the minimal promoter region. Considering the small size of the latter fragment (134 bp), the association of this region with the nuclear matrix is most likely due to the nuclear distribution of the involved transcription factors. Indeed, a number of transcription factors has been shown to be enriched...

Figure 4. In vitro association of the SPRR1A and SPRR2A genes with the nuclear matrix. Rat liver nuclear matrices were incubated with restriction site labelled DNA fragments. Lanes 1 and 4, input DNA; lanes 2 and 5, matrix associated DNA, recovered from the pellet; lanes 3 and 6, soluble DNA. (A) Lanes 1–3 contain SPRR2A gene fragments from pSG-122 digested with HindIII; lanes 4–6 contain SPRR2A gene fragments from pSG-136 digested with HindIII. (B) Lanes 1–3 contain SPRR2A gene fragments from pSG-122 digested with Asp718I (partially) and HindIII. (C) Lanes 1–3 contain SPRR1A gene fragments from pSG-227 digested with HindIII and Bst1107I; note that the intron and promoter fragments have identical gel mobility; lanes 4–6 contain the same plasmid digested with HindIII. Promoter region, intron, promoter region with intron (prom.+intron), minimal promoter region with intron (min. prom.+intron) and vector DNA are indicated.
in the nuclear matrix (63). Both the ISRE and the octamer doublet, unique to the SPRR2A promoter and recognized by ubiquitously expressed transcription factors, could be involved in nuclear matrix association of the promoter region (20, 30). For instance, Oct-1 has been shown to be present in the nuclear matrix (Marenholz, I., Volz, A. and Ziegler, A. (1999) J. Invest. Dermatol., 106, 989–992).

The stimulatory effect of the SPRR2A intron on transcription has been observed in transient transfection. This is in contrast to the effect of most MARs on transcription, which is only apparent after stable integration into the genome (33, 64). The contribution of a MAR to transcriptional regulation is in the order of a 2- to 5-fold increase (51) after stable integration, which is in the same range as observed in transient assays or is even higher. Whether the contribution of the SPRR2A intron to transcription is in the same range as observed in transient assays or is even higher.

**REFERENCES**
