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 SPRR2 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 repetin, 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 SPRR1A (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 epithelium-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 SPRR1A, while an octamer binding site, bound by Oct-1, 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,
the involucrin intron contains an enhancer required for maximal expression of this gene in keratinocytes (34). Furthermore, higher order chromatin structure has been shown to affect transcription (reviewed in 35). In this communication, we have examined the contribution of the introns of the above-mentioned SPRR genes to transcriptional regulation and have identified a nuclear matrix association region (MAR) in SPRR2A.

**MATERIALS AND METHODS**

**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 a layer of lethally 137Cs irradiated mouse 3T3 fibroblasts. For foreskin derived from circumcision and grown in the presence of bovine calf serum (Hyclone, Logan, UT) and primary human fibroblasts (isolated from foreskin) were cultured in DMEM (Gibco BRL, Paisley, Scotland) with 10% bovine 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). 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 30% 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 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 10^7 nuclei/ml.

**Nuclear matrix association experiments**

Nuclei from rat liver cells were isolated as described by Izazarralde 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 10^7 nuclei/ml.

**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 phenylmethylsulfonyl 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 30% 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 C in 0.25 M ammonium sulfate, 1% (v/v) thiglycol, 0.2 M sucrose, 50% (v/v) glycerol] at a density of 10^7 nuclei/ml.
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 which prompted us to examine the SPRR2A intron 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-2). 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 (30). 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 substantially more 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), (Fig. 3A). Such a strong bias for the nuclear matrix associated fraction was not
observed for the related *SPRR1A* gene. We did not observe a significant difference in these distributions between keratinocytes, expressing the *SPRR* genes and HeLa cells or fibroblasts, which do not express these genes; furthermore, the nuclear matrix association of the *SPRR2* gene was not influenced by the keratinocyte differentiation state (data not shown). The quantitative data from two independent experiments with nuclear isolates from primary human keratinocytes, primary human fibroblasts and HeLa cells were depicted in Figure 3B as the average percentage of hybridization to the chromatin fraction which is solubilized by the DNase-1 treatment. As a control for hybridization efficiency, an Alu repetitive element was used, which detects a probe for the *HPRT* MAR. Comparison of the binding affinity of various DNA fragments to rat liver nuclear matrices in *vivo*. 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 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, 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**

In this study we have identified a nuclear MAR which localizes to the promoter and intron of the *SPRR2A* gene. The data
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 SPRR2A promoter activity, for the following reasons: (i) the enhancement of gene expression by insertion of the SPRR2A intron was specific for the SPRR2A promoter and not found for the RSV promoter. This rules out that 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.
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 responsible for the effects we have measured.

Concluding, the present analysis indicates that the intron of SPRR2A enhances transcription of the SPRR2A promoter, yet does not influence terminal differentiation responsiveness. The cooperative binding of the SPRR2A promoter and intron to the nuclear matrix is likely to be the mechanism underlying the observed transcriptional stimulation by the intron.

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