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Evidence for a posttranscriptional effect of retinoic acid on connexin43 gene expression via the 3′-untranslated region

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Abstract ***All-trans*** **retinoic acid (10^{-7} M) induces cell–cell communication and the expression of the gap junction protein connexin43 in mouse F9 teratocarcinoma cells. Previous experiments revealed an increase of mRNA but no change in the transcription of connexin43, suggesting a posttranscriptional mechanism responsible for the regulation of connexin43 gene expression. In transient transfection experiments using an expression vector containing the 3′-untranslated region of the connexin43 gene downstream of the luciferase coding sequence driven by the connexin43 promoter we show here that retinoic acid enhances luciferase activity via the connexin43 3′-untranslated region due to altered stability of the mRNA. Thus, retinoic acid is able to influence connexin43 gene expression at the level of mRNA stability via elements located in the 3′-untranslated region.**

Key words: Connexin43; Gene expression; mRNA stability; Retinoic acid; 3′-UTR

1. Introduction

Gap junctions are cell-to-cell channels, formed by docking and opening of hemichannels in adjacent cells consisting of an array of six subunits, termed connexins, which are coded for by a multigene family (for review, see [1]). Communication via these channels can be regulated by various agents; tumor promoting factors, in most cases, diminish cell–cell communication, whereas agents causing cell differentiation, e.g. retinoic acid, induce cell–cell communication.

Retinoic acid and some of its derivatives exert a variety of biological effects necessary for normal cellular growth and differentiation [2]. The mouse teratocarcinoma cell line F9 is a widely used model for studying mechanisms of differentiation induced by retinoic acid [3].

Connexin43 and connexin26 mRNAs are detectable in stem cells and in differentiated F9 cells, whereas mRNA of connexin32 is not detectable in stem cells [4]. Recently, we have shown that treatment of these cells with retinoic acid in physiological concentrations leads to an increase of intercellular communication associated with an increased amount of connexin43 mRNA but no change in the level of newly transcribed connexin43 mRNA [5]. On this basis, we postulated a posttranscriptional mechanism responsible for the retinoic acid effect. In the present study we examined the possibility of a mechanism via the 3′-untranslated region (3′-UTR) of the connexin43 gene responsible for the induction of connexin43 gene expression by retinoic acid.

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2. Materials and methods

2.1. Cell culture and transfection experiments

F9 cells [6] were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 2 mM glutamine and 20 μg/ml gentamicin on gelatinized plastic tissue culture dishes (Greiner, Frickenhausen, Germany). Differentiation was induced by addition of 1×10^{-7} M *all-trans* retinoic acid (Sigma, Deisenhofen, Germany), dissolved in ethanol. Controls received the same volume of solvent. Transfection was performed with the reagent DOTAP (Boehringer, Mannheim, Germany).

2.2. Luciferase assay

Cells were harvested and luciferase activity was determined from cell extracts using the luciferase assay system (Promega, Madison). The luciferase activities are measured as impulses per second and related to the cellular protein content.

2.3. Plasmids

Fig. 1 shows the constructs named pT81luc, pCxlucSV40, pCxlucCx and pCMVlucSV40. pT81luc and pCMVlucSV40 were kindly provided by Dr. W.A. Schulz (Düsseldorf, Germany) and are described in [7] and [8], respectively. pCxlucSV40 was kindly provided by Dr. R. Werner (Miami, USA) and is described in [9] as pGL2, 1.5 kb. The plasmid pCxlucCx was constructed on the basis of the vector pGEM-luc (Promega, Madison) and contains the promoter of the rat connexin43 gene and the 3′-untranslated region of the mouse connexin43 gene.

2.4. Cloning of the 3′-UTR

The 3′-untranslated region was generated by RT-PCR from F9 cells. The mouse connexin43 sequence [10] was used to construct the oligonucleotides. The 3′-end of the 3′-UTR was first generated by RT-PCR using the oligonucleotide 5′-T(12)-3′ (for reverse transcriptase reaction) and the oligonucleotides 5′-T(12)-3′ and 5′-GCCCATGTATTTGCATCTCAG-3′ for PCR-amplification. The resulting 1.0 kb PCR-fragment was cloned into the vector pGEM-T (Promega, Madison). The 5′-end of the 3′-UTR was generated by RT-PCR using the oligonucleotide 5′-AATCATTTGGTGAGGGTGAGG-3′ for reverse transcription and the oligonucleotides 5′-AATCATTTGGTGAGGGTGAGG-3′ and 5′-CTCCAGCCCTTAGCTATCGTG-3′ for PCR-amplification. This fragment was also cloned into the vector pGEM-T. To generate the complete 3′-UTR both cloned fragments were cut out by the restriction enzymes *Apa*I and *Pvu*I (in the case of the 0.7 kb fragment) or by the enzymes *Pvu*I and *Sph*I (in the case of the 1.0 kb fragment) and ligated via the *Pvu*I site. The complete 3′-UTR was generated by PCR using the oligonucleotides 5′-GTCCTCGAGACAGGCTTGAACATCAAGCTG-3′ and 5′-CTCGAGCTCTTATCTTTTATTATACTAAATT-3′ (designed after sequencing of the 3′-part of the 3′-UTR).

3. Results

Our previous work suggested an effect of retinoic acid on the stability of connexin43 mRNA in the mouse teratocarcinoma cell line F9 [5]. In the present study we turned attention to the 3′-untranslated region (3′-UTR), since most of the known mRNA stability determinants so far have been identified in this region (for review, see [11]). Based on the sequence

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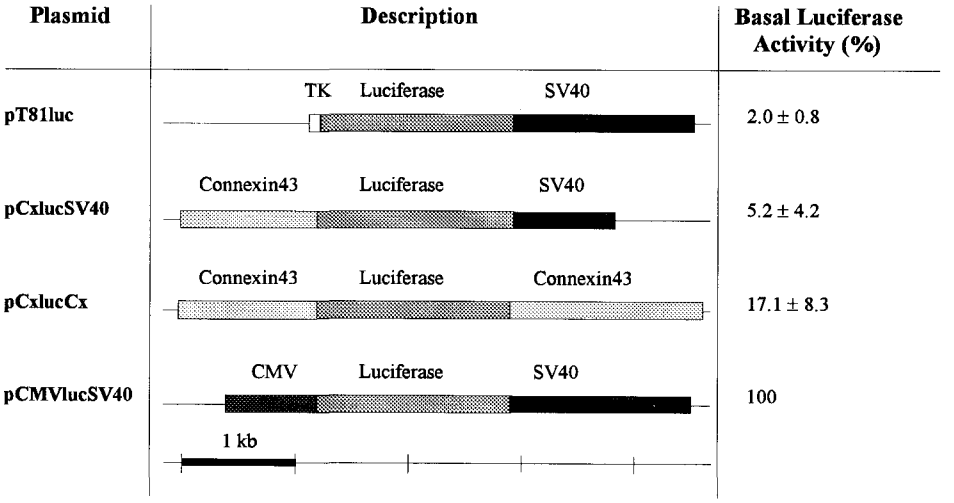

Fig. 1. Luciferase expression vectors used to analyze the effect of retinoic acid on 5′- and 3′-regulatory elements of the connexin43 gene. The coding sequence of the luciferase gene is 5′-flanked by promoters of the genes thymidine kinase (TK), connexin43 or cytomegalovirus (CVM), 3′-flanked by SV40 sequences or the connexin43 3′-UTR. To compare the basal luciferase activities after ten independent transfections, the highest activity (pCMVlucSV40) was set equal to 100%. The other activities are given as means ± S.D.

of the mouse connexin43 gene [10] we cloned the 3′-UTR by RT-PCR using RNA of the cell line F9. The isolated 1.7 kb PCR-fragment was sequenced and contains two potential polyadenylation signals and different AU-rich regions known to confer mRNA instability. To test the function of the connexin43 3′-UTR we prepared the plasmids shown in Fig. 1. Transient transfections of these constructs in F9 cells were performed for the analysis of the connexin43 3′-UTR function. Fig. 1 gives the basal luciferase activity of transfected cells without retinoic acid treatment and shows that the cloned 3′-UTR of the connexin43 gene is able to stabilize the luciferase mRNA.

The results of the luciferase assays after transfection are shown in Fig. 2. As expected, no induction of luciferase activity by retinoic acid occurs via the thymidine kinase promoter (plasmid pT81luc) or the promoter of the connexin43 gene (plasmid pCxlucSV40) and 3′-located SV40 sequences. After transfection of the vector pCxlucCx containing the luciferase coding region and the 5′- and 3′-control regions of the connexin43 gene we observed a 2.6-fold induction of luciferase activity after retinoic acid treatment. Because of the non-response of pCxlucSV40 to retinoic acid (see above) we conclude that retinoic acid enhances luciferase activity via the 3′-UTR of the connexin43 gene in the mouse teratocarcinoma cell line F9. However, the luciferase activity after transfection of the construct pCMVlucSV40 (containing the CMV promoter) can be induced 3-fold by retinoic acid treatment.

4. Discussion

The data shown in Fig. 2 provide evidence for a mechanism by which retinoic acid enhances the stability of the connexin43 mRNA via the 3′-UTR.

Most studies dealing with mRNA stability determinants have identified signals involved in mRNA stability in the 3′-UTRs (for review, see [11]). Transfection experiments have revealed how the 3′-UTR can function as a regulatory determinant independent of the remainder of the mRNA. A few well-characterized elements were identified, some of which are protein binding sites, e.g. 3′-terminal stem-loops from histone mRNAs, AU-rich elements (also termed AUREs), iron-responsive elements (IREs) or long range stem-loop structures of the insulin-like growth factor II (for review, see [11]). Although different factors, like hormones, phorbol esters, cytokines, growth factors or differentiation factors appear to influence mRNA stability, in most cases the mechanisms of actions are not well defined. For example, estrogen is one of the interesting regulating factors of mRNA stability because it stabilizes certain mRNAs, whereas it destabilizes others, even in the same cell type [12,13]. In the case of the connexin43 mRNA AUREs might be involved in the response to retinoic acid. It has been shown that in the case of the interferon mRNA AUREs mediate the glucocorticoid response but do not affect basal expression [14]. We would expect that the effect of retinoic acid on connexin43 mRNA is indirect, because it is known that retinoic acid influences gene expression at the level of transcription by retinoic acid receptors (RARs) and retinoic X receptors (RXRs) which activate target genes through retinoic acid-responsive elements (RAREs) of various promoters in a ligand-dependent pathway [15]. Thus retinoic acid could modulate the transcription of genes coding for proteins involved in posttranscriptional regulation of connex-

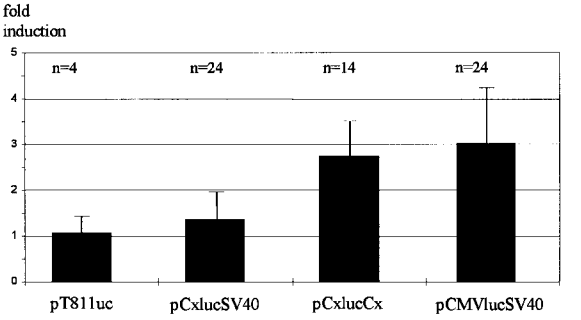

Fig. 2. Induction of luciferase activity by retinoic acid following transient transfection of the constructs given in Fig. 1. Luciferase activity of transfected and solvent treated F9 cells was set to 1, the activity after a 1 day treatment with retinoic acid (10^{-7} M) is given as fold induction ± S.D., n is the number of independent experiments.

in43, e.g. by binding to the 3′-untranslated region of the connexin43 mRNA.

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