Helper activity for gene expression, a novel function of the SV40 enhancer

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ABSTRACT

In this investigation we demonstrate that the enhancer of SV40 possesses an additional function which is a 'helper activity' for a more efficient transfer of viral DNA from the cytoplasm into the nucleus. After DNA transfection into rat-2 cells, the rate of CAT gene expression linked to SV40 promoter/enhancer (pSV2CAT) was approximately 50 fold higher than linked to the tk promoter (pBLCAT2). After direct nuclear microinjection this difference was reduced to a factor of 10. However cytoplasmic injection of the same number of DNA molecules/cell showed again a 50 fold increase for the SV40 promoter/enhancer-CAT construct. This difference was not due to selective degradation of the pBLCAT2 DNA. The 'helper function' did not require the intact 72 bp sequence. In vitro synthesized enhancers lacking certain enhancer motifs (GT-I, TC-II and TC-I sequence) were still effective after cytoplasmic injection whereas an 8 bp deletion (representing a part of the AP-I motif) on the downstream side strongly reduced the helper function after cytoplasmic injection but not the classical transcriptional enhancement after direct nuclear transfer.

INTRODUCTION

Gene expression in higher eukaryotes is mainly regulated at the level of transcription. One of the elements involved in this process are enhancers which are cis-acting DNA segments stimulating transcription from both homologous and heterologous promoters (1,2) and in a fashion mostly independent of their orientation and distance with respect to connected genes (3). The presence of an active enhancer results in an increased density of RNA polymerase II molecules at the linked gene leading to a higher transcription rate (4,5). There is also evidence that host specific molecules interact with specific sequence motifs of the enhancer region (6,7) thereby stimulating the expression of the concerned gene, determining cell and tissue specificity (8,9,10).

The SV40 enhancer is represented by a DNA sequence of 72 bp with multiple motifs connected with transcriptional activation (6). They include the GT-I (11), TC-I, TC-II (12,13), Sph-I and Sph-II motifs (14), and the P-site (15,16). Some of the proteins recognizing these sequences have been identified and characterized as transcriptional activators (11-16). The loss of enhancer function of a mutated or deleted motif can be compensated by duplication of one of the other motifs (17,18).

In this study we find that cytoplasmic injection of CAT constructs containing the SV40 72bp enhancer element showed a 50 fold higher CAT expression than enhancerless CAT constructs. In contrast in direct nuclear injection the difference was only 10 fold. Our results point towards the existence of a so far unknown 'helper function' assignable to the SV40 enhancer which helps DNA to cross the nuclear envelope barrier more easily. Interestingly this 'helper function' did not require an intact 72 bp sequence. In vitro synthesized enhancers

Fig. 1: Four different synthetic DNA sequences were inserted in the Sal I site of pBLCAT2 in front of the tk promoter (tk) and the CAT gene; 72: represents the full SV40 72 bp enhancer sequence, 72-8, 60 and 50 are the truncated enhancers. The deleted bp are represented by the gaps. The recognition sites for transactivator proteins are shown below the sequences.

lacking enhancer motifs such as GT-I, TC-II or TC-I were still effective, increasing the expression of the CAT gene after cytoplasmic injections independent from the 'classical' transcriptional enhancement.

MATERIALS AND METHODS

Cell culture, microinjection and DNA transfection.

For all experiments rat-2 TK⁻ cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 5 % fetal calf serum. Rat-2 cells were plated at 10⁶ cells per 60 mm dishes, 6 hr before each transfection. DNA was transfected by the CaP_i coprecipitation method as described (19). Cells were exposed to CaP_i-DNA for 20 hrs, washed with Ca/Mg-free phosphate-buffered saline and incubated with fresh medium for further 24 hrs. For microinjection rat-2 cells were grown on small glass slides (4mm²) as described (20).

Chloramphenicol acetyl transferase (CAT) assay.

44 hours after transfection the cells were washed with phosphate buffered saline (PBS), scraped off the culture dish with a rubber policeman, centrifuged for 10 min in an Eppendorf centrifuge and resuspended in 200μl 0.25 M Tris-HCl buffer pH7.8. After sonication and incubation at 60°C for 10 min, the cell lysates were cooled on ice and again centrifuged for 10 min. The protein content of the supernatant was measured spectrophotometrically using Bio Rad dye reagent (21). 24 hrs after microinjection the cells on small glass slides

Fig. 2: CAT activity after transfection of pBLCAT2 and pSV2CAT into rat-2 cells. CAT expression measured in cpm per 20 μ g protein 44 hrs after transfection (see material and methods). The data represent mean values of three independent experiments.

were washed with PBS, transferred into Eppendorf tubes, filled with 150 μ l buffer, sonicated and handled as above. The CAT assay was performed by a modification of the standard procedure (22). The reaction time of the cytoplasmic extract with ¹⁴C-chloramphenicol and acetyl-CoA was 17 hrs at 37°C. For separation of the acetylated chloramphenicol (AC) from the unacetylated form the samples were spotted on silica gel plates and chromatographed. Results were quantified by cutting out the bands containing unreacted ¹⁴C-chloramphenicol and AC and the amount of radioactivity was determined by liquid scintillation counting. The input of ¹⁴C-chloramphenicol for each test point was 250.000 cpm. The background for mock transfected cells was about 1000 cpm.

Thymidine kinase activity was analyzed by autoradiography as described elsewhere (23).

DNA constructs.

Standard techniques were used for the treatment of DNA with restriction endonucleases, calf intestine phosphatase, T4 DNA polymerase, T4 polynucleotide kinase, T4 ligase as

Fig. 3: Southern blot analysis of the pBLCAT2 and pSV2CAT DNA reextracted from microinjected rat-2 cells. Ten hrs after injection into the cytoplasm the DNA was reextracted by the modified Hirt method as described (20). The blot was hybridized with nick translated ³²P-CAT DNA. Lane 1: 200 cells microinjected with the pBLCAT2 DNA; lane 2: 100 cells microinjected with the pBLCAT2 DNA; lane 3: the marker pBLCAT2 DNA; lane 4: the marker pSV2CAT DNA; lane 5: 20 cells microinjected with the pSV2CAT DNA; lane 6: 100 cells microinjected with the pSV2CAT DNA.

Fig. 4: CAT activity after nuclear (n) and cytoplasmic (c) microinjection. The number of injected molecules/cell is indicated A: pSV2CAT and B: pBLCAT2. For each experiment 100 rat-2 cells were microinjected and processed for determination of CAT activity 24 hrs later. The data represent the mean values of three determinations, n.d. not demonstrable.

described elsewhere (24). Oligonucleotides were synthesized in vitro with the DNA Synthesizer 380 A (Applied Biosystem Inc.). For construction of recombinant plasmids (Fig.1) four distinct DNA sequences were synthesized in vitro using synthetic oligonucleotides: a) 72: the 72bp SV40 enhancer sequence, b) 60: a truncated form of a) without 12 nucleotides at the 5' end of the sequence, c) 50: a truncated form of a) without 22 nucleotides at the 5' end of the 72bp enhancer, d) 72-8: the 72bp region missing 8 nucleotides at its 3' end. All oligonucleotides were synthesized with Sal I linkers (5' GTCGAC) at both ends and with a Hpa II site (5' CCGG) at the 3' and 5' ends and the constructs of c) and d) contain an additional Xba I site (5' TCTAGA) at the 3' end. The DNA segments were inserted into the Sal I site of pBLCAT2 in front of the tk-promoter

Fig. 5: CAT assay 24 hrs after microinjection. 100 rat-2 cells were injected into the nucleus (n) or the cytoplasm (c). 1: pBLCAT2 (1000–2000 molecules/cell); 2: pSV2CAT (1000–2000 mol./cell); 3: pBLCAT2 (200–400 mol./cell); 4: pSV2CAT (200–400 mol./cell); 5 : control (mock-injected rat-2 cells).

(Fig.1). Large scale plasmid preparation and purification of supercoiled DNA by CsCl gradient centrifugation were performed. The size and the number of the DNA inserts were verified on polyacrylamide gels using the ^{32}P -labeled Sal I, Sph I and Xba I digests.

The pSV-tk construct is a derivative of the pTKSO DNA (25). It contains the HSV-tk gene driven by the early SV40 promoter/enhancer segment (Hpa II/Bgl I fragment with Sal I linker). To obtain the pSPT19-tk DNA, the HSV-tk gene with its own promoter (Pvu II fragment of pHSV (BRL)), was inserted into the Hinc II site of the pSPT19 DNA (Pharmacia).

RESULTS AND DISCUSSION

We have analyzed the biological activity of constructs containing the coding sequence of chloramphenicol acetyl transferase (CAT) gene with either the HSV-tk promoter (pBLCAT2) (Fig.1) (26) or the early SV40 promoter segment (pSV2CAT) (9,22). In these experiments DNA was transferred into rat-2 cells by transfection or microinjection and CAT activity in cell extracts was measured (see material and methods).

After transfection of the pSV2CAT construct in amounts as low as 0.01 μg DNA/dish CAT activity was observed (2000–3000 cpm), which increased 10 fold with 0.1 μg DNA/dish and reached a saturation at 5–10 μg (Fig.2). Similar results were reported earlier (27,28). In contrast pBLCAT2 DNA was almost 50 fold less active (Fig.2) such that 0.5 μg DNA/dish of this construct was necessary to obtain CAT activity comparable to that with 0.01 μg DNA/dish of pSV2CAT DNA. Saturation was not yet demonstrable at 10 μg pBLCAT2 DNA/dish.

On the assumption that the efficiency of cytoplasmic DNA uptake during transfection is the same for pSV2CAT and for pBLCAT2 DNA we conclude that the SV40 promoter is responsible for enhanced CAT expression in rat-2 cells. Indeed in experiments involving DNA reextraction from transfected or microinjected cells and Southern blot analysis, the signal intensity for pSV2CAT and pBLCAT2 DNAs was very similar as shown in fig.3. The molecular basis of DNA uptake during transfection is not yet well understood. It has

Fig. 6: (^3H)-thymidine incorporation into rat-2 TK⁻ cells after cytoplasmic (c) or nuclear (n) microinjection of (A) pSV-tk and (B) pSPT19-tk. (^3H)-thymidine was added to the medium directly after injection. The cells were fixed at the indicated time points and processed for autoradiography. The numbers of injected cells are counted as 100%. The equations for the linear regressions of the four time courses are indicated. The data are mean values from three independent injection experiments.

been demonstrated that about 7.5% of the total DNA added per culture dish enters the cells as part of the calcium phosphate complex, but the majority of the cells accumulated the transfected DNA in the cytoplasm and only a few cells contained detectable amounts of the DNA in the nuclei (29). This suggests that the movement of the DNA from the cytoplasm to the nucleus is one of the limiting steps for the expression of transfected plasmids. Interestingly cells which are 'transfection resistant' internalize the DNA but do not express the trans DNA molecules (30). This resistance is limited to the DNA transfection

Fig. 7: CAT expression after nuclear (n) and cytoplasmic (c) injections of **A** pBLCAT2-72; **B** pBLCAT2-60; **C** pBLCAT2-50 and **D** pBLCAT2-72-8 into rat-2 cells, n.d. not demonstrable. (see legend of Fig. 3).

procedure because these cells support gene expression after direct micro-injection (31).

Taking advantage of the fact that only microinjection allows the transfer of test molecules in known amounts and desired intracellular localization we analyzed the biological activity of the pSV2CAT and pBLCAT2 DNA after either nuclear or cytoplasmic injection. CAT activity was detectable after nuclear microinjection of as few as 2–4 pSV2CAT and 20–40 pBLCAT2 DNA molecules (Fig. 4 A,B). Increasing the number of injected DNA molecules resulted in increased CAT activity. After the transfer of 1000–2000 molecules/cell the measured CAT activity was similar for the pSV2CAT and pBLCAT2 DNA (Fig. 4 and 5). Thus, in comparison to the tk promoter, the SV40 promoter shows up to a 50 fold higher activity in transfection experiments and only a 10 fold higher in intranuclear microinjection which requires to be explained. The apparent discrepancy can be explained by an additional effect of the SV40 promoter in transfection experiments. In such a scheme, the putative ‘helper function’ would involve increasing the efficiency of DNA uptake from the cytoplasm to the nucleus. To test this idea we microinjected pSV2CAT and pBLCAT2 into the cytoplasm of rat-2 cells. After cytoplasmic microinjection the CAT activity for both constructs was lower than that after intranuclear injection (Fig. 4). Comparing the effect of intracellular site for the pSV2CAT DNA, 10 fold more molecules were required in cytoplasmic microinjection than in nuclear injection in order to obtain the same CAT activity (2000–3000 cpm). For the pBLCAT2 construct a 50 fold higher DNA concentration was required (1000–2000 molecules/cell). In conclusion, the results of cytoplasmic

microinjection are similar to those obtained after DNA transfection namely that CAT expression was clearly driven 50 fold higher with the SV40 than with the tk promoter .

The 'helper function' is also demonstrable as a delay in the onset of gene expression. We microinjected the HSV-tk gene driven either by its own promoter (pSPT19-tk) or by the SV40 promoter (pSV-tk) either into the cytoplasm or into the nuclei of rat-2 TK⁻ cells with a multiplicity of 20–40 molecules/cell. In Fig.6 the time course of tk expression after nuclear injection is compared to that after cytoplasmic injection. The pSV-tk injected into the nucleus induced tk expression two hours faster than the same construct, injected into the cytoplasm (Fig.6 A). This time lag in expression between nuclear and cytoplasmic injection was three times longer with the pSPT19-tk (Fig.6 B). Moreover the slopes for the time courses of expression do not differ substantially for nuclear injections of both constructs and for the cytoplasmic injection of pSV-tk. The cytoplasmic injection of the pSPT19-tk however results in a slope of expression, which is 4 to 5 times lower than that of the others. The difference in time lag as well as in the slope of tk expression strongly argue for a function of the SV40 in the uptake of DNA from cytoplasm into the nucleus. In situ hybridization experiments have to prove this working hypothesis.

To test whether the postulated 'helper function' can be attributed to the early SV40 enhancer region, the 72 bp element was synthesized in vitro with Sal I linkers at both ends (Fig.1) and inserted into the Sal I site of the pBLCAT2 DNA. This construct termed pBLCAT2–72 was microinjected into rat-2 cells and the CAT activity was monitored. Insertion of the 72 bp enhancer sequence upstream of the tk promoter and intranuclear injection of 2–4 DNA molecules/cell caused a 10 fold increase in CAT expression, which is comparable to that of pSV2CAT (Fig.4 A and 7 A). It is notable that after cytoplasmic injection of 20–40 molecules CAT expression was detectable as it was for pSV2CAT. Furthermore this induced CAT expression was similar to the results obtained after nuclear injections of 2–4 molecules. That means, a 10 fold higher concentration of DNA is required for cytoplasmic injections to get comparable CAT expression to the nuclear injections. From these results we conclude that the 72 bp enhancer element is responsible for the 'helper function' in addition to the transcriptional enhancement.

To analyze whether partial deletion of the enhancer sequence causes loss of the helper function truncated enhancer sequences were synthesized and inserted into the Sal I site of the pBLCAT2-DNA upstream of the tk-promoter in the same orientation as in their original position in the SV40 genome (see Fig.1 and material and methods). The truncated 60 bp enhancer sequence in pBLCAT2–60 lack the first 12 bp from the upstream part of the enhancer which includes the transcriptional activator GT-I binding motif (11) (containing the core sequence (32)) and a part of the TC-II motif (12,13). The 50 bp sequence in pBLCAT2–50 lacked further 10 bp comprising the rest of the TC-II motif and a part of TC-I. The 60 bp enhancer was integrated into the plasmid DNA as a three times repetitive sequence, while the 50 bp was repeated four times (see material and methods). The microinjection data summarized in Fig.7B,C clearly demonstrate that pBLCAT2–60 and pBLCAT2–50 induced a stronger enhancement of CAT activity than pBLCAT2–72 in both nuclear and cytoplasmic microinjection. These data are consistent with results of others that covalently linked Sph-I/ Sph-II motifs (33,34) or the Ap-I motif (35) alone when inserted as repeated segments upstream of reporter genes give increased enhancement. The 'helper function' was again realized, as showed by high CAT activity after cytoplasmic injections.

The last truncated enhancer analyzed was the 72-8 sequence. The 8 bp deletion at the downstream position of the 72 bp enhancer includes part of the cellular transactivator Ap-

I binding motif (Fig.1). We observed that this deletion did not significantly affect the gene activity after nuclear microinjection (Fig.7 D). In contrast to that, microinjection of pBLCAT2-72-8 into the cytoplasm caused a substantially decreased expression of CAT compared to all other constructs with full or truncated enhancer elements. This decreased expression is slightly above the expression gained after cytoplasmic injection of the enhancerless construct pBLCAT2 (Fig.4 B), suggesting that the AP-I motif is essential for the 'helper function'. However our preliminary results indicate that the AP-I site alone is not sufficient the helper function.

CONCLUSION

In eukaryotic cells different kinds of macromolecules have to migrate from one cell compartment into another across cell membranes which do not allow free diffusion. The nuclear envelope constitutes such a compartment boundary through which RNA and protein molecules are selectively translocated. Precursor RNAs, synthesized in the nucleus are retained there whereas the processed forms are transported into the cytoplasm for their various tasks. Proteins synthesized in the cytoplasm and destined for the nucleus, to constitute structural elements or to take part in regulation of gene expression, are passaged into the nucleus while cytoplasmic proteins are excluded (36). It has been shown that this translocation process is highly dependent on specific signal sequences in the case of proteins (36) and of tRNA (37).

For DNA, no cytoplasm-nucleus transport mechanism seems to be needed but nevertheless viral DNA molecules have to pass through the cytoplasm into the nucleus in order to allow gene expression. Therefore it is not surprising that viral DNA molecules contain sequences which mediate a more efficient uptake of the DNA from the cytoplasm into the nucleus. Since all DNA transfer techniques except microinjection outwit the plasmamembrane of the cells but not the nuclear envelope only the direct intranuclear injection allowed differentiation between the enhancement of transcription and the 'helper function'.

So far it is not clear how the helper activity is mediated. However it is tempting to speculate that this requires the interaction of specific DNA sequences with a cellular protein(s). That a DNA-protein complex can pass through membranes was demonstrated recently for the DNA transport from the cytoplasm into the mitochondria (38).

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