A ubiquitous enhancer shared by two promoters in the human aldolase A gene

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ABSTRACT

The human aldolase A gene is transcribed from three different promoters, which are all clustered within a 1.6 kbp DNA domain. Two of these, P_N and P_H , are ubiquitous and seem to be co-regulated in most tissues while the third one, P_M , is specific to adult skeletal muscle. We investigated the sequences involved in the ubiquitous activity of the P_N and P_H promoters of the human aldolase A gene. Deletion analysis, performed by transient expression assays of chloramphenicol acetyltransferase reporter genes in human HepG2 hepatoma cells, indicated that P_H activity results from the interaction of an upstream activating region with two distinct core promoters. The upstream activating region was able to stimulate transcription from the HSV tk promoter as efficiently as the SV40 enhancer in all cell types tested. It appears, therefore, to be a strong ubiquitous enhancer. DNaseI footprinting revealed protections covering sequences scattered along the enhancer, including Sp1 and AP1 motifs. Importantly, we found that this enhancer was also necessary to activity of the other ubiquitous promoter of the aldolase A gene, P_N . These studies demonstrate that expression of the human aldolase A gene is mediated by a complex interplay of enhancer and promoter elements.

INTRODUCTION

Alternative promoters are frequently used to express the same gene product at different stages of development or in different cell types (1). For example, the human aldolase A gene is transcribed from three tandem promoters, ' P_H ', ' P_N ' and ' P_M ' (2,3,4). Each of these leads to the synthesis of mRNAs with different 5' ends, but with a common coding sequence (see Figure 1 for a description of the 5' region of the aldolase A gene). P_H -derived mRNAs are abundant in a wide variety of tissues. However, in adult heart and skeletal muscle they are five to ten fold more abundant than in other tissues. The P_H ubiquitous promoter is, therefore, modulated in a tissue-specific fashion. As frequently reported in promoters of housekeeping genes, P_H initiates transcription at multiple sites: a 5' start site (at position +3114) and two rather heterogeneous 3' start sites (at positions +3160 and +3174)(2). Two putative TATA boxes could be associated to these sites (2,3), one is upstream of the 5' start site

(TTTAAAA spanning positions +3088 to +3094) and the other is upstream of the 3' start sites (TAAAT spanning positions +3147 to +3151). Interestingly, increased expression of P_H in muscle is associated with a switch in the relative utilization of its different start sites (4). The P_N promoter seems to be coregulated with P_H since P_N -derived mRNAs are distributed similarly to P_H -derived mRNAs. However, P_N -derived mRNAs are always approximately twenty fold less abundant than P_H -derived mRNAs (4). Finally, P_M -derived mRNAs are highly specific to adult skeletal muscle.

The fact that the three promoters of the aldolase A gene are all clustered within a 1.6 kbp DNA domain suggests that these promoters might be sharing some transcriptional regulatory elements.

In this study, we investigated the sequences involved in the ubiquitous expression of the P_H and P_N promoters of the human aldolase A gene. A deletion analysis of sequences involved in the high level P_H activity was performed, demonstrating that it resulted from three distinct contributions: two autonomous core promoters and one strong ubiquitous enhancer. In order to understand how such a ubiquitous enhancer functions in different cell types, enhancer subdomain analysis and DNaseI footprinting were performed. These studies suggest that both similar and different transactivating proteins are required for full enhancer activity in different cell types. In addition, we have found that the low-level P_N promoter activity also relies upon this enhancer, which is, therefore, shared by the two ubiquitous promoters of the aldolase A gene.

MATERIAL AND METHODS

Plasmid constructions

Plasmid DNA manipulations were done according to standard procedures (5). (i) **Deletion analysis plasmids.** All test plasmids were derived from two plasmids, pNMHCAT and pHCAT (4), which were obtained by insertion of aldolase A sequences from the genomic clone lambda 6A (2) upstream of the CAT gene in poCAT (6). (ii) **Enhancer analysis plasmids.** The indicated fragments were purified from pHCAT and subcloned into pBLCAT2 (7). ptkSV40 was obtained by inserting the SV40 enhancer from pSV2CAT (8) into SmaI cut pBLCAT2. (iii) **Riboprobe plasmids.** Riboprobe plasmids were obtained by subcloning appropriate aldolase A fragments from the test plasmids in pKS (Stratagene). (iv) **DNaseI footprinting analysis probe.** The PstI/+2693-DraI/+3089 fragment was subcloned

HepG2 (9), 3T6 and F9 cells (10) were grown at 37°C in DMEM (Gibco) supplemented with 10% fetal calf serum (Gibco). C2C12 (11) cells were grown in DMEM supplemented with 20% fetal calf serum. Samples of plasmids (15 µg), which included 7.5 µg of test plasmid, 0.5 µg of the internal transfection efficiency control plasmid—pRSVLuci (12)—and 7 µg of carrier KS plasmid (Stratagene), were introduced into the cells by the calcium phosphate coprecipitation technique. Fourty to forty-eight hours after transfection, the cells were harvested and assayed for luciferase (12) and CAT activities (4). Measured CAT activities were normalized by the corresponding measured luciferase activities.

Uniformly radiolabeled antisense riboprobes were synthesized using template riboprobe plasmids which had been previously

DNaseI protection assays

Isolation of nuclear proteins from subconfluent cultures of HepG2, F9, and C2C12 cells and DNaseI footprinting were performed as described (13,14).

An important activating region is located just upstream of the H₂ exon

In order to find DNA elements important for activity of the P_H promoter, various domains of the aldolase A gene, with varying 5' ends and a common 3' end as depicted in Figure 1, were

Figure 1. Deletion analysis of the P_H promoter in human HepG2 hepatoma cells. The 5' region of the human aldolase A gene is depicted in the top of the figure. Each of the promoters P_N, P_M, and P_H leads to the synthesis of mRNAs containing different 5' exons (N1 and N2, M, and H respectively) spliced to a common coding sequence (starting at the ATG codon in exon C1). Arrows represent start sites of transcription. The start sites of P_N are very heterogeneous (4) and are represented by a single arrow for the sake of clarity. Restriction sites used in the construction of test plasmids are also shown at their position along the sequence of the gene. (All nucleotide co-ordinates follow the numbering in the published sequence.(2)) Relative CAT activity of test plasmids in transfected HepG2 cells: a fixed amount of the pRSVLuciferase expression vector (12) was cotransfected along with the test plasmid and provided an internal control for transfection efficiency; normalized CAT activities of test plasmids, representing the mean of two to four independent experiments, were expressed by comparison to the normalized CAT activity of the pHCAT plasmid whose activity was set at 100%.

cloned in front of the chloramphenicol acetyltransferase (CAT) reporter gene and transfected into human HepG2 hepatoma cells to test for their transcriptional activity. Figure 1 shows that the strongest activity was obtained with pHCAT, a construct whose 5' end is at the PstI site located 420 bp upstream of exon H. This plasmid has previously been found to be as active as the pSV2CAT plasmid in HepG2, LTK- and 3T6 cells (4 and our own data). Domains extending 5' from the PstI site were all less active than pHCAT but still remained at least half as active. This

Figure 2. Mapping of the start sites of transcription from pHCAT and pHCAT Δ NarI in transfected HepG2 and 3T6 cells. RNAs from human HepG2 and murine 3T6 cells transfected with plasmids pHCAT or pHCAT Δ NarI were analyzed by a RNase protection assay using the antisense riboprobe described at the bottom of the figure. The size of riboprobe fragments protected by correctly initiated transcripts is also given (in nucleotides (nt)). The arrows along the gel indicate the position of such fragments. Lane Ct is a negative control with RNAs from control non transfected 3T6 cells. None of the bands designated by the three upper arrows was detected in control HepG2 cells (data not shown). Lane M contains size markers (sizes shown in nucleotides). The asterisks indicates bands which correspond to protection by different types of RNAs. Because the Aval/+2995-NarI/+3101 fragment was removed by the internal deletion in pHCAT Δ NarI, any transcript initiated at or upstream of the NarI/+3101 site in this plasmid provided riboprobe protection starting at this site only. The asterisks, therefore, indicate, in lanes corresponding to cells transfected with pHCAT Δ NarI, protection by transcripts initiated upstream of the NarI site.

suggests that no major regulatory elements were present 5' of the PstI site. Shorter domains were all less active, and the shorter was the domain, the weaker was the activity. This demonstrated that an important activating region is located just upstream of the H exon, between the PstI site at position +2693 and the NarI site at position +3101.

Initiation of transcription in transfected plasmids follows the complex pattern described *in vivo*

We wanted to determine whether initiation of transcription in transfected plasmids followed the complex pattern described *in vivo* (see introduction and Figure 1). The 5' ends of the CAT transcripts were, therefore, mapped in transfected human HepG2 cells and in transfected murine 3T6 fibroblasts by RNase protection assays. Two constructs were selected: pHCAT, because it was the most active one, and pHCAT Δ NarI, because this internal deletion construct derived from the pHCAT plasmid contains all three start sites, but does not include the upstream putative TATA box whose deletion potentially affected the localization of initiation of transcription.

In HepG2 cells transfected with pHCAT, three protected fragments of approximately 347, 301 and 287 nucleotides (nt) were found, corresponding to initiation at the correct sites (figure 2, lane HepG2/HCAT).

Figure 3. Analysis of minimal constructs derived from the P_H promoter by transfection into HepG2 cells. The arrangement of start sites (arrows) and putative TATA boxes (diamond-shaped boxes) at the H exon region is represented in the top part of the figure. 3' and 5' deletions progressively isolating the 5' start site and the 3' start sites were tested for their transcriptional activity by transfection into HepG2 cells as described in Figure 1. CAT activities of the test plasmids depicted on the left are given relative to that of pAval-HCAT whose activity was set at 100% and represent the mean of two to four independent experiments. The weakest construct, pBvI-HphI-CAT generated CAT activities which were 4 to 8 times higher than those generated by poCAT (6), the parental plasmid which has no eukaryotic promoter. One could observe that progressive 5' deletions had the same effect in the group of constructs with 3' ends at the HphI site as in the group of constructs with 3' ends at the FspI site. Comparison of constructs with similar 5' ends but with 3' ends at the HphI site within exon H or at the FspI site downstream of exon H, showed that the HphI-FspI fragment provided a one to two fold increase in CAT activity. Whether this resulted from the presence of some weakly activating intragenic elements, or from the splicing of the CAT transcripts was not further investigated.

Figure 4. Mapping of the start sites of transcription from minimal constructs in transfected HepG2 cells. RNAs from control non-transfected HepG2 cells (lane Ct) or from HepG2 cells transfected with indicated plasmids were analyzed by a RNase protection assay using the antisense riboprobes described at the bottom of the figure. The size of riboprobe fragments protected by correctly initiated transcripts is also given. The arrows indicate the position of such fragments. Lane M contains size markers. **(A)** Mapping of the start sites of transcription from minimal constructs containing the 5' start sites in transfected HepG2 cells. **(B)** Mapping of the start sites of transcription from minimal constructs containing the 3' start sites in transfected HepG2 cells. The SV40 enhancer was introduced behind the CAT gene in pBbvI-HphI-CAT creating pBbvI-HphI-SV40. The asterisks indicates protection by transcripts initiated upstream of the BbvI site (see comment in legend to figure 2). Endogenous aldolase A mRNAs containing the longer 172 nt H exon were detected here as a 91 nt protected fragment (not shown on the figure). The aldolase A mRNAs containing the shorter 126 nt and 112 nt H exons were not detected under the conditions used here. Lane M contains size markers.

In contrast, in HepG2 cells transfected with pHCAT Δ NarI, only the two shorter fragments were found (figure 2, lane HepG2/HCAT Δ NarI). The longer fragment corresponding to initiation at the 5' start site could not be detected. Deletion of the NarI/+2844-NarI/+3101 fragment from pHCAT, therefore, had a drastic qualitative effect: it virtually abolished initiation of transcription at the 5' start site, and, yet, it did not affect initiation of transcription at the 3' start sites.

In 3T6 cells transfected with pHCAT, three protected fragments of approximately 347, 301 and 287 nucleotides (nt) were found, corresponding to initiation at the correct sites. Unexpectedly, protected fragments of approximately 172, 126 and 112 nt were also found, which were similar to the fragments protected by the endogenous aldolase A mRNAs in HepG2 cells. In 3T6 cells transfected with pHCAT Δ NarI, two protected fragments of 301 and 287 nt were found, corresponding to initiation at the 3' start sites, together with unexpected 126 nt- and 112 nt-long fragments. We believe that these unexpected protections, rather than resulting from initiation of transcription at unexpected sites, resulted from splicing of the correctly initiated CAT RNA species, which could have occurred between the donor

splice site at the end of exon H and some cryptic acceptor splice site. This fortuitous splicing of CAT constructs did not affect the conclusions drawn from the deletion analysis, as examined by performing a new deletion analysis with a series of plasmids containing aldolase A domains with 5' ends as in Figure 1, but with 3' ends at the HphI site in exon H so that they did not contain the donor splice site (data not shown).

These results showed that initiation of transcription in transfected plasmids followed the complex *in vivo* pattern. However, in pHCAT Δ NarI, initiation at the 5' start site could not be detected. This suggested that in P_H, at least two distinct 'core'/'basal' promoters (15) mediated initiation of transcription, one of which would consist in the upstream TATA box associated with the 5' start site.

Two distinct core promoters mediate initiation of transcription

In order to further establish this finding, we analyzed, by transfection into HepG2 cells, a series of 'minimal' constructs containing the 5' start site and the 3' start sites progressively isolated one from the other. Figure 3 shows that two short and non-overlapping fragments of DNA—a 78 bp AluI-BbvI

ENHANCER ANALYSIS PLASMID		RELATIVE CAT ACTIVITY			
A		HepG2	3T6	C2C12	F9
1	1	1	1
10.5	6	7.1	9.9
8.1	4.7	4.9	7.5
10.1	3.4	5.6	n.d.
6.1	n.d.	n.d.	n.d.
12	8.9	12.2	10.4
B
6.7	3	4.5	9.4
3.6	3.1	2.2	3.6
4.7	3	3.3	8.4
1.5	3.4	1.2	3
1.4	1.1	2.2	2.1

Figure 5. Enhancer activity of the upstream activating region from the P_H promoter. (A) A PstI-DraI fragment encompassing the PstI-AluI upstream activating region defined by the deletion analysis was tested for enhancer activity. It was cloned at different positions relative to the tk promoter in the pBLCAT2 plasmid (7) and the resulting plasmids were tested in various cell lines. The SV40 enhancer was cloned downstream of the tk promoter in pBLCAT2 and provided a positive control for stimulation of the tk promoter. For each particular cell type, normalized CAT activities, representing the mean of two to four independent transfection experiments, are given as fold stimulation of the normalized CAT activity generated by the tk promoter in pBLCAT2. n.d., not determined. (B) Independent enhancer activity of subdomains of the upstream activating region of the P_H promoter. Various fragments of the PstI-DraI fragment were tested for independent enhancer activity in various cell lines. For each particular cell type, relative normalized CAT activities are expressed as mentioned above.

fragment, containing the upstream putative TATA box and the 5' start site, and a 63 bp BbvI-HphI fragment containing the downstream putative TATA box and the 3' start sites—were sufficient to generate significant CAT activity.

Mapping of the 5' ends of aldolase A-CAT mRNAs was performed in transfected HepG2 cells in order to examine the initiation sites used in these constructs. Figure 4A shows that the AluI-BbvI fragment was sufficient for specific initiation at the 5' site. Figure 4B shows that, only very little specific initiation could be observed from the 3' start sites with pBbvI-HphICAT. The small Bbv-HphI fragment was, therefore, tested for its ability to mediate site specific initiation of transcription when stimulated by a remote enhancer. Addition of the SV40 enhancer to pBbvI-HphICAT, at a position 1.6 kb downstream of the BbvI-HphI fragment, led to a ten fold increase in CAT activity (data not shown) and specific initiation at the two 3' sites was clearly observed (Figure 4B). The BbvI-HphI fragment was, therefore, sufficient for specific initiation at the 3' sites.

From this series of experiments, we conclude that in the P_H promoter, initiation of transcription is mediated by two distinct core promoters, the AluI-BbvI and the BbvI-HphI fragments, respectively.

The activating region is a strong ubiquitous enhancer

The activating region found upstream of the H exon was next tested for its ability to stimulate transcription from a heterologous promoter. For that purpose, a PstI/+2693-DraI/+3088

fragment—encompassing the upstream activating region mapped by the deletion analysis shown in Figure 1—was cloned in both orientations, both upstream and downstream of the HSV tk promoter in the pBLCAT2 plasmid (7). As a control for tk promoter stimulation, the SV40 enhancer was cloned downstream of the tk promoter in the same plasmid. After transfection into HepG2 cells, it was found that the PstI-DraI fragment enhanced activity of the tk promoter in all positions tested, and that the levels of stimulation were comparable with that provided by the SV40 enhancer (see Figure 5A). This fragment will be referred to from now on as the H enhancer.

Since *in vivo* P_H is active in a wide variety of cell types, we tested whether this would also be the case for the H enhancer. Cells of different embryonic origin, possibly showing a large variety in their respective batteries of transcriptional factors, were tested: 3T6 murine fibroblasts, C2C12 murine myoblasts and undifferentiated F9 embryonal carcinoma cells. As shown in Figure 5A, the H enhancer was active in all cases, and again, the levels of stimulation were comparable with those provided by the SV40 enhancer.

We next wanted to know whether the H enhancer could be divided into independent subdomains and what would be their enhancer activity in different cell types. Constructs containing various H enhancer subdomains were tested. Figure 5B shows that each subdomain was able to stimulate transcription from the tk promoter in all cells. Fold stimulation was always lower than for the whole enhancer and it did not vary markedly from one cell type to another.

Footprinting analysis of the H enhancer

To determine the sequences which interacted with nuclear proteins, footprint analysis of the H enhancer was performed. Nuclear extracts from three of the cell lines in which enhancer activity was examined were employed in order to compare footprint patterns. In every case, footprints were observed, scattered all along the enhancer. As shown in figure 6A and 6B, each of the previously examined enhancer subdomains were bound by nuclear proteins. These proteins, therefore, potentially accounted for the independent enhancer activity observed. Comparison of the footprint patterns obtained with extracts of different cell-origin indicated that the footprinted regions were roughly similar in all cell types examined. However, the precise protection pattern in each footprinted region varied from one cell type to another: it was either identical (as in footprinted regions 2, 3 and 4), slightly different (footprinted region 1) or totally different (footprinted region 5). Protected regions showed sequence homology to transcriptional control elements found in other genes (Figure 6C and Discussion). In particular, footprinted region 5 contained an AP1 motif. Competition experiments were done in which increasing quantities of unlabelled 'PK' double-stranded oligonucleotide, containing a high-affinity AP1 binding site (16), were included in the mixture of probe and nuclear proteins. They showed that in HepG2 cells, the lower part of footprint 5 was due to AP1 binding (data not shown). The upper part of footprint 5 was not affected, which indicated it was bound by another protein complex. No effect of competition with this oligonucleotide could be observed on the footprint pattern in F9 cells. This is in agreement with observations made in other laboratories that only a little AP1 activity can be detected in F9 undifferentiated cells (16,17). We conclude that the H enhancer was bound by both similar and different proteins in different cell types.

Figure 6. DNaseI footprinting analysis of the H enhancer. The PstI-DraI fragment was labeled at the 3' end by filling in with Klenow DNA polymerase. After a 15 min incubation on ice without (lane —) or with indicated μ g quantities of nuclear extracts from HepG2 and F9 cells (A) or C2C12 cells (B), samples were submitted to limited DNaseI digestion and after separation run on a 6% denaturing polyacrylamide gradient gel. Lanes G + A show products of a sequencing reaction of the labeled fragment used to measure the nucleotide positions of the protected regions. On the right side of the gels are indicated the position of the sites delimiting the various enhancer subdomains analyzed in Figure 5B. On the left side of the gels are schematized the footprinted regions. Boxes indicate areas of protection from DNaseI digestion. Arrows indicate DNaseI hypersensitive nucleotides. These features of the footprint patterns were either specific to a particular cell type (and indicated to the extreme left under the HepG2, the F9 or the C2 legends), or common to all cell types (and indicated under the C2 + HepG2 + F9 legend). (C) Footprinted regions are related to known cis-acting elements. Partial nucleotide sequence of the enhancer is reproduced. Boxed sequences represent footprinted regions in HepG2 cells and are numbered as in A. Dots indicate homologies in these footprinted regions to known cis-acting elements: the Sp1, 'GGTGG' (31), and AP1 (43) motifs. Footprinted region 4 showed no obvious homology to previously described cis-acting elements.

The H enhancer is necessary to activity of the N promoter

Coordinate expression of P_N - and P_H -derived mRNAs was reported in the various tissues examined, with P_N -derived mRNAs always being approximately twenty fold less abundant than P_H -derived mRNAs (4). This observation suggested that these promoters might be sharing some regulatory DNA elements.

We, therefore, examined whether the H enhancer was also necessary to the P_N promoter activity. A hybrid gene was constructed by fusing the 3' end of the human β globin gene to the 5' region of the human aldolase A gene cloned in the laboratory. Two internal deletions were also prepared in order to test for the role of the H enhancer in P_N activity: one removed the H exon and start sites and left the H enhancer intact, the other removed both the H exon and start sites and two-thirds of the H enhancer (see Figure 7A). Activity of P_N in the constructs was assayed by determining the relative levels of P_N -derived aldolase A/ β globin transcripts after transfection into murine C2C12 cells. An internal control for transfection efficiency was provided by the cotransfection of a fixed amount of the pSV2CAT plasmid together with each test plasmid. Figure 7B shows that in their normal context, both P_N and P_H promoters were active (lanes E14). Deletion of P_H promoter sequences was not detrimental to activity of P_N (lanes E14 Δ 1). In contrast, further deleting aldolase A sequences from the hybrid gene and now removing enhancer sequences up to the NarI/+2844 site led to a drastic decrease in activity of the P_N promoter (lanes E14 Δ 3). These findings showed that the H enhancer was necessary to activity of the P_N promoter.

DISCUSSION

P_H promoter structure: 2 distinct core promoters

We showed that two short DNA fragments containing respectively the 5' start site and the two 3' start sites of the P_H promoter were able to function as autonomous core promoters, that is, they contained information sufficient for basal level transcription at the correct initiation sites (Figures 3 and 4). This suggests that the switch observed in the relative utilization of start sites of the P_H promoter in muscle (4) is due to differential stimulation of the core promoters by activating sequences in different cell types. Differential start site usage has been shown to occur in other genes, at different developmental stages or in different tissues (18) or in response to environmental stimuli (18, 19, 20). It could also be due to the existence of multiple, differentially stimulated core promoters.

In fact, the mouse metallothionein-I promoter has been shown to have two core promoters, one being TATA-dependent and metal-inducible while the other is TATA-independent and not metal-inducible (18). In the H promoter, the differential stimulation of the two core promoters could be due to the fact that they contain different TATA boxes. Recent studies have, indeed, provided strong evidence for functional heterogeneity among TATA boxes: TATA boxes from different promoters were shown to differ in their ability to support stimulation of transcription by enhancer sequences (21) or by E1A (22).

P_H promoter structure: 1 strong, ubiquitous enhancer

The strong activity of the P_H promoter was found to be due to the presence of an important activating region just upstream of the H exon (Figure 1). This result is in agreement with previous studies (23). We further investigated the properties of this region.

Figure 7. The H enhancer is necessary to activity of the other ubiquitous aldolase A promoter, P_N . (A) An aldolase A/ β globin hybrid gene was prepared in order to examine simultaneously the activities of P_H and P_N . The 5' region of the human aldolase A gene, from position +1 of the published sequence (see Figure 1) to the BspmII site from the intron in between coding exons C2 and C3 (3), was fused to the 3' end of the human β globin gene, from the RsaI/+12360 site in the second intron to the PstI/+13550 site located beyond the polyadenylation signal (44). The resulting hybrid gene is contained within a plasmid named pE14. Two plasmids containing deleted hybrid genes were derived from pE14: pE14 Δ 1 and pE14 Δ 3, in which were removed, a NarI/+3101-BglII fragment containing the H exon and start sites, and, a NarI/+2844-BglII fragment containing the H exon and two-thirds of the H enhancer, respectively. (B) RNAs from C2C12 cells transfected with 20 μ g of test plasmid (pE14, pE14 Δ 1, and pE14 Δ 3, respectively) and 2 μ g of internal control plasmid (pSV2CAT, 8) were analyzed by a RNase protection assay using a mixture of the two riboprobes described at the bottom of the figure. One of these riboprobes allowed to detect transcripts containing exon N2 as a 60 nt protected fragment. The other riboprobe, which was identical to that used in figure 4B, allowed to detect: (i), transcripts containing the longer 172 nt H exon as a 91 nt protected fragment, (ii), transcripts of the CAT gene, generated by the internal control plasmid, as a 250 nt protected fragment. Lane C2C12/ Ct and lane 'RNAs are negative controls using the same mixture of probes and control non-transfected C2C12 cells RNAs or yeast 'RNAs. The strong band in lane C2C12/Ct was artefactual and probably resulted from incomplete digestion and/or insoluble material in this sample as evidenced by the persistence of radioactive material in the slot. Lane H/ Ct is a positive control for the riboprobe targeted at the N2 exon, using this riboprobe alone and HepG2 RNAs. Comparison of this lane with the negative controls shows that only the 60 nt band designated by the arrow corresponded to exon N2 containing transcripts.

We demonstrated that it constitutes a strong enhancer in a wide variety of cell types: HepG2 hepatoma cells, C2C12 myoblasts, undifferentiated F9 embryonal carcinoma cells. It was also active in retinoic acid differentiated F9 cells as well as in primary cultures of newborn rat neuronal cells (H. Skala and our own unpublished data).

Although many viral enhancers have been described that are also active in a large number of cell types, to our knowledge, only one other 'ubiquitous' enhancer has been described in a mammalian gene so far (24). The existence of ubiquitous enhancers in such housekeeping genes contrasts with the frequent existence of separate tissue-specific enhancers in genes expressed in a limited number of different tissues (25,26).

How does a ubiquitous enhancer function in different cell types?

The footprint analysis illustrated in Figure 6 showed that there were sites of interaction with DNA binding proteins scattered all along the enhancer. The protected regions were evenly distributed among the enhancer subdomains previously examined, showing a reasonable correlation between the independent enhancer activity of the various subdomains and its inclusion of footprinted sequences (Figures 5B and 6).

These regions of the aldolase A enhancer, defined by functional and footprint analyses, contained sequences related to cis-acting elements found in other genes. Footprinted regions 1, 2 and 5 contained sequences related to the binding site for the ubiquitous transcription factor Sp1, which is consistent with the fact that these sequences were protected in all cell types. Recent studies showing that, like other cis-acting elements (27), the Sp1 motif is able to bind a whole family of transcription factors (28, 29,30) may explain why footprinted region 1 showed a footprint pattern which differed slightly in F9 cells from that observed in HepG2 and C2C12 cells. Footprinted region 3 contained a sequence highly homologous to the GGTGG motif, which was recently described in the β globin LCR and was also reported to bind ubiquitous proteins (31). Finally, numerous reports of the contribution of AP1 sites to basal level gene transcription (32,33) make it likely that the AP1 site in footprinted region 5 is important for enhancer activity in HepG2 and C2C12 cells. In contrast, even when important quantities of nuclear extracts were used, no protection was observed over the AP1 site contained in footprinted region 5 in F9 cells. Taken together, these considerations suggest that the H enhancer may be recruiting both ubiquitous and cell-specific transcription factors in order to function in different cell types. This has been shown to be the case with ubiquitous viral enhancers (34). To test this hypothesis, the functional significance of the various interactions described needs to be directly examined, for instance by testing the effect of mutations in specific footprinted regions in different cell types.

The H enhancer is shared by P_H and P_N promoters

Figure 7 shows that in aldolase A/ β globin hybrid genes, as long as the H enhancer sequences were present, P_N was active. When approximately two-thirds of the H enhancer sequences were deleted, P_N activity was virtually abolished. These findings show that P_N is also dependent on the H enhancer, and provide a functional explanation for how P_N - and P_H -derived mRNAs are coordinately expressed in a wide variety of tissues. Why the remaining third of the H enhancer, corresponding to the PstI-NarI/+2844 fragment which was able to stimulate the tk promoter at least 3 fold in the same cells (Figure 5B), was not

sufficient to stimulate efficiently P_N , is at present unclear. It could be that the whole enhancer is required to efficiently stimulate a weak promoter such as P_N and that there is a synergy in the collaboration of the different enhancer subdomains. Such a synergy would not be observed with a relatively much stronger promoter, such as the tk promoter. The synergy in the collaboration of domains A and B of the SV40 enhancer provides an example of such a mechanism (35).

Enhancers shared by several transcription units have already been reported. In some cases, it has been shown that the shared enhancer stimulates simultaneously both target promoters (36, 37, 38). In other cases, it has been shown that the shared enhancer stimulates alternatively each of the target promoters at different stages of development or in different tissues (39, 40, 41, 42). In this regard, it will be of interest to examine whether the H enhancer also stimulates the third aldolase A promoter, P_M , in adult skeletal muscle. We are currently studying lines of transgenic mice harbouring the aldolase A/ β globin hybrid genes described in Figure 7A in order to examine this question.

In summary, the present study demonstrates an unusually complex interplay of enhancer and promoter elements in the aldolase A gene. We showed that a ubiquitous enhancer, which is as potent as the SV40 enhancer, is responsible for stimulation of three different targets: two core promoters, which mediate initiation of transcription in the P_H promoter, and the P_N promoter. This enhancer interacts with multiple DNA binding proteins in HepG2, F9 and C2C12 cells, whose roles in the stimulation of each of the different promoters it will be interesting to determine.

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