The utrophin gene codes for a large cytoskeletal protein closely related to dystrophin. Its transcription is driven by a TATA-less promoter. Here we analyzed 40 kilobases of the 5′ end region of the utrophin gene searching for new utrophin regulatory elements in muscle cells. By transient transfection of utrophin genomic fragments in front of a reporter gene, we identified a new enhancer that maps downstream of the transcription start site within the second intron and co-localizes with a DNase I-hypersensitive site. By deletion analysis it was mapped to a sequence of 128 base pairs that retains the whole activity. Linker scanning mutagenesis showed that most of the enhancer sequence is essential for its transcriptional activity. Binding analysis with nuclear cell extracts demonstrated that the enhancer regulatory elements, identified by mutagenesis, are protected from DNase I digestion. Because utrophin can functionally substitute dystrophin, the identification and characterization of new regulatory elements provide new targets for possible therapies of Duchenne muscular dystrophy aiming at the up-regulation of the utrophin expression in muscle cells.

Duchenne muscular dystrophy (DMD)⁴ and Becker muscular dystrophy are two forms of a fatal X-linked disease affecting 1 in 3500 newborn males and are caused by alterations or non-expression of the dystrophin gene in muscle. The dystrophin has been shown to be localized in the internal sarcolemmal membrane, where it is necessary for the assembly of a large transmembrane glycoprotein complex of skeletal and cardiac muscle (1, 2). The dystrophin provides an important link between actin cytoskeleton and the extracellular matrix. The lack of dystrophin in DMD results in the absence of dystrophin-associated glycoprotein and muscle degeneration, which leads to a fatal muscle necrosis. The utrophin gene (also named dystrophin-related gene) is an autosomal homologue of dystrophin (3). Utrophin is transcribed in a large mRNA of 13 kb containing the 5′ end of human utrophin gene, obtained from the Resource Center/Primary Data Base of the German Human Genome Project, were partially digested with the restriction endonuclease Sau9AI ligated to SuperCos1 cosmids vector arms and packaged using the Gigapack III XL packaging extract (Stratagene) according to the manufacturer’s description. The cosmid library obtained was screened by hybridization with a DNA probe encompassing the first thousand base pairs of the human utrophin promoter sequence (EMBL accession number X95523). This probe was obtained by polymerase chain reaction from human genomic DNA of a healthy individual using the following oligonucleotides: G5 (GGATAAGCTTGGAACAGGCCTATAACAGATGG) and G6 (GGATTTCAGACATCGGCTCTGGAGCCAGGGGCT). The positive clones E22 and H1A were partially digested with the restriction endonucleases BamHI and BglII, and the resulting fragments were cloned in front of the utrophin promoter driving the expression of the reporter gene CAT (plasmid UP). The overlapping fragments obtained had variable dimensions from 300 to 7000 bp and covered all the genomic region of 40 kb.

The subclones of C14 were produced by restriction digestion as indicated in Fig. 3, A and C. The plasmid TK has been described as pBLCAT2 (16). TK-DUE was obtained positioning the 128-bp fragment of the enhancer DUE (downstream utrophin enhancer) upstream of the herpes simplex virus TK promoter in pBLCAT2. The promoter and enhancer inserts of all reporter constructs were confirmed by automated dyeodeoxynucleotide sequencing (Applied Biosystems, Inc., model 377).

Cell Culture, Transfection, and CAT Assay—Mouse C2C12 cells, human RD rhabdomyosarcoma cells (ATCC CCL-136) and HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). After transfection, the differentiation of C2C12 cells into myotubes was allowed by changing growth medium to Dulbecco’s modified Eagle’s medium containing 2% FBS.

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‡ The abbreviations used are: DMD, Duchenne muscular dystrophy; kb, kilobase(s); bp, base pair(s). DUE, downstream utrophin enhancer.

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The subclones of C14 were produced by restriction digestion as indicated in Fig. 3A or by polymerase chain reaction using internal primers (see Fig. 3, B and C). The plasmid TK has been described as pBLCAT2 (16). TK-DUE was obtained positioning the 128-bp fragment of the enhancer DUE (downstream utrophin enhancer) upstream of the Herpes simplex virus TK promoter in pBLCAT2. The promoter and enhancer inserts of all reporter constructs were confirmed by automated dyeodeoxynucleotide sequencing (Applied Biosystems, Inc., model 377).

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horse serum (17). All recombinant plasmids were purified using the Qiagen plasmid kit (Qiagen Inc., Chatsworth, CA). For each transfection experiment, cells were seeded at 4 × 10^5 cells/60-mm dish and transfected 18 h later with an equal molar amount of reporter plasmid (maximum 5 μg) and 0.75 μg of pRSVβgal as transfection efficiency control, using LipofectAMINE™ Reagent and following the manufacturer’s indications. 4 h later, serum-free DNA-containing medium was replaced by fresh growth medium, and the cells were harvested after 24–48 h. Cell extracts and CAT assays were performed as described previously (18).

**Nuclear Extracts and Footprinting**—Nuclear extracts were obtained as described previously (19). The DNA probe for DNase I footprinting was prepared by 5’ end labeling of the insert of clone C14,13 (sense strand) using T4 polynucleotide kinase (New England Biolabs). The probe was purified on 5% polyacrylamide gel (1 strand) using T4 polynucleotide kinase (New England Biolabs). The DNA probe for DNase I footprinting was suspended in 50 μl of footprinting buffer (10 mM Tris-HCl, pH 8, 50 mM NaCl, 10 mM KCl, 2 mM MgCl_2, 1 mM dithiothreitol, 0.1% Nonidet P-40, 10% glycerol, 0.04 mg/ml poly(dI-dC), 1 mg/ml bovine serum albumin) for 20 min at room temperature. 1 μl of DNase I (1 unit/μl) in footprinting buffer containing 5 mM CaCl_2 was added, and incubation was continued for 1 min at room temperature. DNase I digestion was stopped by the addition of stop buffer (192 mM sodium acetate, 32 mM EDTA, 0.14% SDS, 64 μg/ml sonicated calf thymus DNA). Samples were then extracted with phenol–chloroform, phenol, ethanol precipitated, and resuspended in 5 μl of sequencing loading buffer (U. S. Biochemical). After denaturation at 95 °C for 4 min, samples were subjected to 6% urea gel electrophoresis and autoradiographed.

**DNase I Hypersensitivity Assay**—The DNase I hypersensitivity site assay was performed as described previously (20). Briefly, 2 × 10^6 RD cells were collected in 1 ml of phosphate-buffered saline buffer and then resuspended in 4 ml of RBS buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl_2). The suspension was placed in a Dounce homogenizer (pestle B), and the homogenate was layered over 6 ml of RBS, 0.5 ml of digestion buffer (15 mM Tris-HCl, pH 7.5, 15 mM NaCl, 60 mM KCl, 3 mM MgCl_2, 0.5 mM dithiothreitol, 0.25 mM succrose). All the digestion were performed at 37 °C for 10 min with 1–10 μg/ml of DNase I and stopped by addition of DNase I stop solution (25 μl of digestion buffer (15 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl_2) and 0.75 μl of DNase I (1 unit/ml)). DNA was purified, subjected to restriction enzyme digestions, blotted, and hybridized with the BglII-BgIII fragment.

**RESULTS**

**Identification of Enhancer Elements within the Utrophin 5′ Region**—To identify new utrophin regulatory elements, we cloned a region of about 40 kb surrounding the utrophin promoter from the YAC clones ICRFy9001H10124 and ICRFy900-E03E3 containing the 5′ end of the human utrophin gene. The cosmid library generated was screened by hybridization with a 1000-bp DNA probe containing the utrophin promoter (13), and the positive cosmid clones E22 and H1A have been selected (Fig. 1A). The intronic DNA fragment contained the whole transcriptional activity of the promoter (UP) alone into mouse muscle C2C12 cells (black bars) and in human RD cells (gray bars). The means of three independent experiments are shown.

**Characterization of the Downstream Utrophin Enhancer**—To restrict to the minimum the DNA fragment containing the enhancer activity, we generated internal deletions of the BgIII-BgII fragment using restriction endonuclease sites present within this DNA segment and subcloned the resulting fragments in form of the utrophin promoter driving the expression of the reporter gene CAT. Of the seven clones generated, three retained the enhancer activity by transient transfection in RD cells (Fig. 3A). Further deletions from both extremities (Fig. 3, B and C) revealed that a small DNA fragment of 128 bp (from +8815 to +8942) contained the whole transcriptional activity (clone 14.17; Fig. 3C). This enhancer element was named DUE.
In transient transfections of RD muscle cells DUE was fully active either in front of the utrophin or the heterologous thymidine kinase promoter in both orientations (Fig. 4). Although in the present study we were interested in analyzing DUE activity in muscle cells, utrophin is expressed ubiquitously (9, 10). We therefore tested whether DUE activated transcription also in HeLa cells. Transient transfection of HeLa cells revealed that DUE enhanced by about 8-fold the utrophin promoter (Fig. 4B) and thus showed similar activity in HeLa, C2C12 myoblasts, and RD cells. We also tested DUE activity during muscle differentiation in C2C12 myoblasts and myotubes. Transient transfection of DUE in C2C12 showed no significant differences in its transcriptional activity between myoblasts and differentiated myotubes (data not shown).

**Cis-acting Elements and trans-Acting Factors Binding to DUE**—To further characterize the sequence elements of DUE, we mutated the enhancer sequence by linker scanning. In each mutant 6 nucleotides were substituted with the sequence coding for the restriction site XbaI. 16 mutations spanning the whole 128-bp enhancer were generated (Fig. 5A). The transcriptional activity of mutants was analyzed by transient transfection in RD muscle cells. 11 mutations of 16 affected the enhancer activity, indicating that a large part of this DNA sequence is necessary for the enhancer function (Fig. 5B). The mutations clustered mainly within two subregions covering both enhancer extremities. To localize transcription factors binding to DUE, DNase I footprinting experiments of the enhancer were carried out with nuclear extract from RD muscle cells. Within the enhancer element the footprinting revealed an extensive protection covering most of the sequences whose mutations affected the enhancer activity in RD muscle cells (Fig. 6).

**FIG. 2.** Identification of chromatin DNase I-hypersensitive site in the second intron of the utrophin gene. A, schematic view of the BgIII-BgIII fragment containing the hypersensitive site. Numbers above indicate the positions relative to the more upstream utrophin transcription start site (13). The XhoI-HindIII fragment used as probe is indicated by a white bar. B, Southern blotting analysis of DNA extracted from nuclei of RD cells treated with various amounts of DNase I (see “Experimental Procedures”), purified, digested with BgIII, and hybridized with the probe indicated in A. The hypersensitive site is indicated in each panel with an arrow.

**FIG. 3.** Deletion analysis of the BgIII-BgIII fragment containing the utrophin downstream enhancer DUE. Numbers above indicate the positions relative to the utrophin transcription start site. The + sign indicates full enhancer activity, the − sign indicates loss of enhancer activity. A, schematic representation of the clones obtained by endonuclease digestion. B and C, deletion clones obtained by polymerase chain reaction DNA amplification. Clone 14.17 contains the shortest DNA fragment with enhancer activity in transfection experiments.

**FIG. 4.** DUE is active in both orientations and in front of the utrophin promoter as well as on the TK promoter. A, transcriptional activity of DUE linked to the thymidine kinase (TK) promoter compared with the activity of the TK promoter alone. B, transcriptional activity of DUE in rhabdomyosarcoma RD (gray bars) and in HeLa cells (white bars) linked to the utrophin promoter in the forward (DUE) or in the reverse (DUE-Rev) orientations. The transcriptional activity of the enhancer was compared with the activity of the promoter alone (UP). The means of three independent experiments are shown.

**DISCUSSION**

In muscle, utrophin is expressed at low levels as its transcription is limited to the myonuclei of postsynaptic sarcoplasm (12, 21). The utrophin promoter contains a functional N box recognized by the Ets-related transcription factor GA-binding protein, which has been demonstrated to confer a promoter response to heregulin (14). Although the utrophin promoter can be up-regulated by trophic factors such as heregulin, the levels of induction obtained are quite modest, probably because there
is only one heregulin responsive element on the promoter. Efficient transcription requires the synergistic action of multiple factors bound at distinct sites upstream or downstream of the promoter (for a review see Ref. 22). With the aim of finding additional regulatory elements outside of the characterized promoter sequence, we analyzed the genomic region of the utrophin gene spanning for about 40 kb surrounding the transcriptional start site. In this study we report the identification and characterization of a new regulatory element of the utrophin gene.

The utrophin enhancer was identified by transient transfection of a library containing the 5′ end of the utrophin gene into the rhabdomyosarcoma cell line RD. This approach revealed two DNA fragments potentially important for transcriptional activation of the utrophin gene in muscle cells. One enhancer, which increased expression driven by the utrophin promoter only in mouse muscle C2C12 cells, was not further characterized. A second enhancer increased expression driven by the utrophin promoter of about 7-fold in both muscle cell lines used in our initial screening. In human RD cells this enhancer showed to co-localize with a DNase I-hypersensitive site, thus suggesting that the utrophin downstream enhancer corresponds to chromatin accessible region. This utrophin enhancer behaves as a classical enhancer that works in both orientations and enhances the transcription of the utrophin promoter as well as the heterologous thymidine kinase promoter. We did not observe significant differences of DUE activity between undifferentiated myoblasts and differentiated myotubes, suggesting that the rather modest increase in utrophin expression during myogenic differentiation previously observed by others (23) is not due to regulatory elements localized in DUE. Transient transfections in HeLa revealed that DUE activity is not limited to muscle cells. Although a more careful analysis both in vitro and in vivo should be done to study the tissue specificity of DUE, this result could be predicted because the utrophin gene is expressed ubiquitously. As the same regulatory elements can be recognized and activated by different factors in different cell types we focused our analysis on muscle cells. DUE is a compact enhancer of only 128 bp. This is the minimal size required for the assembly of a nucleosome structure and roughly corresponds to the dimensions of the well known human interferon β enhancer (see Refs. 24 and 25 and reference therein). Linker scanning mutagenesis showed that most of the DUE sequence is essential for its activity in muscle cells, suggesting that several nuclear factors recognize its sequence. This was confirmed by the binding analysis with nuclear proteins, which showed a good correlation between the

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**Fig. 5.** Linker scanning mutagenesis of DUE. A, sequence of human DUE is indicated. Numbers indicate the relative positions of the first and last nucleotides of DUE with respect to utrophin transcriptional start site. Gray bars under the sequence indicate the linker scanning substitutions of the wild type sequence with the sequence recognized by XbaI restriction enzyme. Names of the mutants are indicated. B, transcriptional activity of linker scanning mutants linked to the utrophin promoter compared with the activity of the promoter alone (UP) and the enhancer wild type (DUE). The means of three independent experiments are shown.

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**Fig. 6.** DNase I footprinting analysis of DUE. The DNase I digestion of the free (lane F) and bound (lane B) probes are compared. Lane M, Maxam and Gilbert sequence reaction as marker. Gray bars indicate the relative positions of linker scanning mutants as described in Fig. 5. The white bars indicate the presence of cleavage protection from DNase I endonuclease activity. Black dots indicate the presence of new DNase I-hypersensitive sites on the bound probe.
mutations that affect the enhancer activity and the protected region. Similar to what happens on the interferon $\beta$ enhancer, most of DUE sequence is protected from digestion with DNase I, suggesting also that on this enhancer the correct assembly of several nuclear factors is required for its full activity. To fully characterize DUE-dependent transcriptional regulation it is therefore mandatory to identify all the nuclear factors binding to it. Several studies described examples of transcription factors activated in response to a variety of external signals, which upon activation bind to their cognate sequence on regulatory elements. Therefore, once these specific factors recognizing DUE will be known, it will be straightforward to imagine a scenario in which is possible to influence the correct assembly of the enhanceosome on DUE in muscle cells and hence its activity. Search for known transcription factor consensus sequences on DUE, performed with the program TFSEARCH 1.3 with minimum of score of 85, suggests that factors like AP-1, GATA, and AML-1 could bind to DUE. Thus, these factors are good candidates to be part of the enhanceosome complex. Further studies are necessary to address the identity of the factors binding to DUE and their possible activation in cardiac and skeletal muscle cells.

The results described in this study provide new information about the transcriptional regulation of the utrophin gene useful for the understanding of its regulation in muscle cells and represent a first step forward drug design with the aim of the up-regulation of the utrophin gene in dystrophic patients.

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