Cure of Xenografted Human Carcinomas by BR96-Doxorubicin Immunoconjugates

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Immunoconjugates (BR96-DOX) were prepared between chimeric monoclonal antibody BR96 and the anticancer drug doxorubicin. The monoclonal antibody binds an antigen related to Lewis Y that is abundantly expressed at the surface of cells from many human carcinomas; it has a high degree of tumor selectivity and is internalized after binding. BR96-DOX induced complete regressions and cures of xenografted human lung, breast, and colon carcinomas growing subcutaneously in athymic mice and cured 70 percent of mice bearing extensive metastases of a human lung carcinoma. Also, BR96-DOX cured 94 percent of athymic rats with subcutaneous human lung carcinoma, even though the rats, like humans and in contrast to mice, expressed the BR96 target antigen in normal tissues.

Although chemotherapy is an effective treatment for selected human tumors, only modest progress has been made for the majority of carcinomas, including carcinomas of the breast, lung, and colon. The introduction of the monoclonal antibody (mAb) technology in the 1970s raised hopes that tumor-specific mAbs could be used to target antitumor agents and provide more effective therapy (*1*). Various immunoconjugates, in which antibodies were used to target chemotherapeutic drugs (*2–7*), or plant and bacterial toxins (*8–10*) have been evaluated in preclinical models and found to be active in vitro and in vivo. However, activity was usually assessed against newly implanted rather than established tumors and was typically superior only to matching, but not optimal, doses of unconjugated drug. Although conjugates have been described with antitumor activity against established tumors that was superior to that of an optimal dose of unconjugated drug, the therapeutic index was low, and superior activity was achieved only at or near the maximum tolerated dose (MTD) of the conjugate (*7*). The results of clinical studies of drug and toxin conjugates have also been disappointing, particularly for solid tumors (*11–14*).

As an attempt to improve antibody-directed therapy of human carcinomas, we have conjugated the mAb BR96 with the anticancer drug doxorubicin (DOX). As used in the studies described here, BR96 is a chimeric (mouse-human) variant of the murine BR96 mAb (*15*), is of the human immunoglobulin G1 (IgG1) isotype, and was produced by homologous recombination (*16*). BR96 binds to a tumor-associated antigen that is closely related to Lewis Y (Le^y) and is abundantly expressed (>200,000 molecules per cell) on human carcinoma lines. According to immunohistology, BR96 binds the majority of human carcinomas of the breast, lung, and colon (*15*). Although BR96, like essentially all mAbs to human tumors, is not truly tumor-specific, it offers advantages over most other antibodies to Le^y (*17–20*). First, BR96 is more tumor selective and the normal tissues to which it binds primarily comprise differentiated cells of the esophagus, stomach, and intestine as well as acinar cells of the pancreas (*15, 21*). Second, BR96 is rapidly internalized into lysosomes and endosomes after binding to cells expressing the antigen (*15, 22*). We used the latter characteristic to design conjugates that rapidly release DOX after antigen-specific internalization. We prepared the conjugates by linking the DOX derivative maleimidocaproyl doxorubicin hydrazone to BR96 or control immunoglobulins (Fig. 1). These conjugates incorporate a thioether linker, which imparts acceptable stability in plasma (*23, 24*), and an acid-labile hydrazone bond (*7, 25, 26*) which liberates DOX once it is internalized into the acidic environment of lysosomes and endosomes.

The BR96-DOX conjugate demonstrated antigen-specific activity in vitro and was 8- to 25-fold more potent than nonbinding (IgG-DOX or SN7-DOX) conjugates against carcinoma lines that expressed the BR96 antigen. BR96-DOX was much less active against cells that did not bind BR96 (*27*).

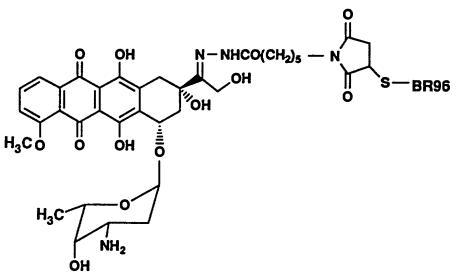

Fig. 1. Structure of the BR96-DOX immunoconjugate.

We evaluated the antitumor activity of BR96-DOX against human lung adenocarcinoma L2987, colon carcinoma RCA, and breast carcinoma MCF7 growing as subcutaneous transplants in athymic mice (Fig. 2). According to immunohistology, the degree of binding of BR96 to cells from these carcinoma lines was similar to that of biopsy material from human carcinomas of the same respective types (*21*). Controls included untreated mice and mice that received DOX (at doses optimized to produce maximal antitumor activity in each model), unconjugated BR96, mixtures of BR96 and DOX, and DOX conjugated to either normal human IgG or the control mAb SN7. Doses of DOX and mAb are presented as milligrams per kilogram of body weight per injection. Therapy (three treatments 4 days apart) started 14 to 28 days after tumor transplantation when tumors were well established. Treatment with BR96-DOX consistently cured most mice bearing L2987 (Fig. 2A) or RCA (Fig. 2B) tumors and complete and partial tumor regressions were produced against MCF7 tumors (Fig. 2C). Equivalent doses of nonbinding IgG-DOX or SN7-DOX had no effect against these tumors. Although optimal doses of DOX delayed the growth of small L2987 tumors (50 to 100 mm^3) and MCF7 tumors, regressions or cures were not observed. Alone, DOX was not active against established RCA tumors either in terms of tumor growth delay or regressions; however, BR96-DOX cured 78% of the treated mice. BR96-DOX also produced 56% cures and 22% complete and 22% partial regressions of lung tumors which were 250 to 800 mm^3 in size at the start of therapy (Fig. 2D). In contrast, antitumor activity was not observed after treatment with an optimal dose of DOX.

Table 1 summarizes the tumor regression rates after treatment with various doses of BR96-DOX, IgG-DOX, DOX, and mixtures of mAb and DOX against established L2987 and RCA tumor xenografts. When administered at equivalent DOX doses of ≥5 mg/kg (three injections 4 days apart), BR96-DOX produced long-term cures in 72 to 100% of mice (n = 281) bearing L2987 tumors. In the RCA colon tumor model, which was not sensitive to unconjugated DOX, BR96-DOX administered at equivalent DOX doses of ≥10 mg/kg (three injections 4 days apart) cured 72 to 100% of mice (n = 48). Mice cured of L2987 or RCA tumors remained alive and tumor-free for more than 1 year with no indication of

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side effects. Administered at equivalent doses, BR96 was not active against established tumors (either in terms of tumor growth delay or regressions), and the delay of tumor growth produced by mixtures of BR96 and DOX was equivalent to that of DOX administered alone.

Contrary to our expectations, cells lacking BR96 expression were not detected after treatment with BR96-DOX (*27*). Also, cells obtained from tumors that grew back after BR96-DOX induced regression were as sensitive in vitro to DOX as the parental cell line; the IC_{50} (concentration required to produce 50% inhibition of [^{3}H]thymidine incorporation) was 0.4 ± 0.1 μM and 0.3 ± 0.2 μM of DOX for treated and parental, respectively. These cells were also as sensitive to BR96-DOX as the parental cell line with IC_{50} values of 2.6 ± 0.8 μM and 2.7 ± 0.5 μM equivalent DOX for treated and parental, respectively. These data suggest that it may be possible to successfully re-treat tumors with several rounds of BR96-DOX therapy.

The MTD (equivalent DOX dose) of the BR96-DOX conjugate (three injections 4 days apart) was 20 mg/kg administered intraperitoneally (ip). When administered intravenously (iv), the MTD was ≥10 mg/kg. This was the maximum dose that could be given iv because of the constraints of injection volume. At the doses tested, there was no difference in the antitumor activity of BR96-DOX whether administered ip or iv. At doses of BR96-DOX (Table 1) equivalent to ≥5 mg/kg of DOX [BR96 (≥250 mg/kg)], more than 70% of treated animals were cured of established L2987 tumors. In fact, BR96-DOX was active at doses as low as a DOX equivalent of 1 mg/kg. The BR96-DOX conjugate was, therefore, active at a dose equivalent to 1/20th of its MTD. These data demonstrate the broad range of therapeutic doses that were achieved with BR96-DOX. The MTD of unconjugated DOX (8 mg/kg iv and 4 mg/kg ip) was lower than that of the BR96-DOX conjugate. Unconjugated DOX, administered ip at the MTD, did not inhibit the growth of established L2987 tumors. When administered iv at the MTD, DOX produced a delay in tumor growth but no tumor regressions, and if the dose was reduced to 50% of the MTD, DOX had no effect (*7*). In contrast, activity equivalent to that of an optimal dose of DOX (8 mg/kg) was achieved at a dose of BR96-DOX of 1 mg/kg; the BR96-DOX conjugate produced antitumor activity comparable to that of an optimal dose of unconjugated DOX at 1/8th of the equivalent DOX dose. In summary, the BR96-DOX conjugate was more active, had a much broader range of therapeutic doses, and was more potent than unconjugated DOX.

Although many immunoconjugates have been described that are active against subcutaneous tumors (*2*–*7*), activity against established disseminated disease has not, for the most part, been observed (*2*, *28*). Cells from L2987 lung carcinoma were selected in vitro for their ability to grow as multicellular spheroids. When we injected these cells iv into athymic mice, tumors developed at various sites, including lymph nodes, lung, spleen, liver, brain, and subcutaneously, and ascites was formed in some animals. Cells obtained from these disseminated tumors expressed the BR96 antigen, as shown by indirect immunofluorescence, in amounts similar to those of the L2987 cell line and subcutaneous tumors. Mice inoculated with L2987 spheroids and treated (14 mice per group) with BR96-DOX (DOX equivalent of 8 mg/kg administered as three injections 4 days apart) had an increased median survival time (MST) (MST of >200 days) relative to that of control mice (MST of 85 days) or mice treated with an optimal dose of DOX (MST of 140 days).

In another experiment, therapy was delayed until mice displayed extensive disseminated disease, defined as ≥0.5 g of visible tumor burden (Fig. 3). The disease in these

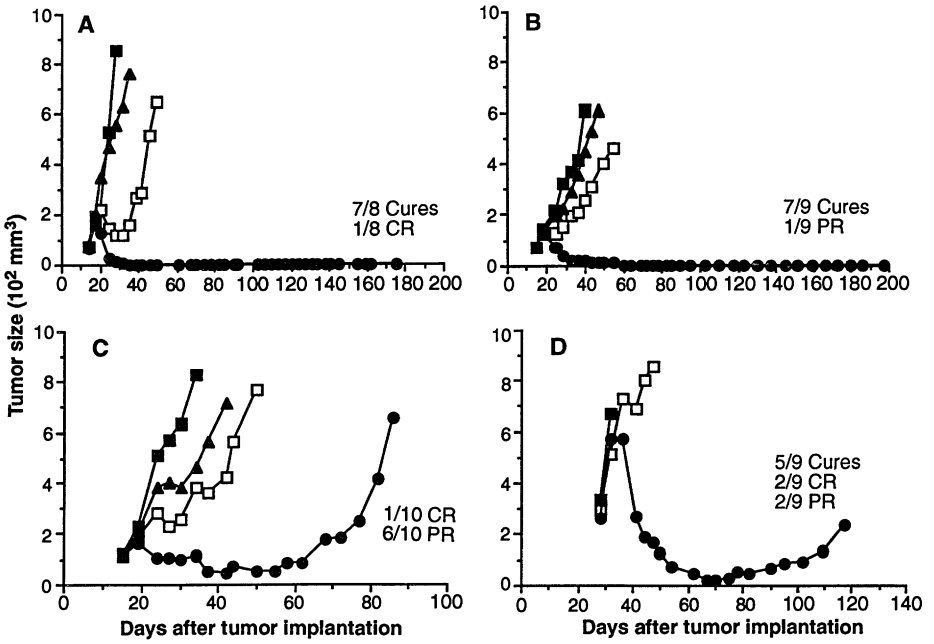

Fig. 2. Antigen-specific antitumor activity of BR96-DOX conjugates. Partial tumor regression (PR) reflects a decrease in tumor volume to ≤50% of the initial tumor volume; complete tumor regression (CR) refers to a tumor that for a period of time is not palpable; and cure is defined as an established tumor that is not palpable for a period of time ≥10 tumor volume doubling delays (the time in days that it takes for control tumors to double in size). Data are presented as median tumor size. (**A**) Xenografts from L2987 lung tumor 50 to 100 mm^3 at the initiation of therapy. Control animals (■); animals treated with BR96-DOX [DOX (5 mg/kg)] (●), IgG-DOX [DOX (5 mg/kg)] (▲), or optimized DOX (8 mg/kg) (□) 14, 18, and 22 days after the tumor was implanted. (**B**) Xenografts from RCA colon tumor 50 to 100 mm^3 at the initiation of therapy. Control animals (■); animals treated with BR96-DOX (10 mg/kg) (●), IgG-DOX (10 mg/kg) (▲), or optimized DOX (8 mg/kg) (□) 16, 20, and 24 days after the tumor was implanted. (**C**) Xenografts from MCF7 breast tumor 100 to 125 mm^3 at the initiation of therapy. Control animals (■); animals treated with BR96-DOX (5 mg/kg) (●), IgG-DOX (5 mg/kg) (▲), or DOX (6 mg/kg) (□) 14, 18, and 22 days after the tumor was implanted. (**D**) Xenografts from L2987 lung tumor 250 to 800 mm^3 at the initiation of therapy. Control animals (■); animals treated with BR96-DOX (8 mg/kg) (●) or DOX (8 mg/kg) (□) 28, 32, and 36 days after the tumor was implanted.

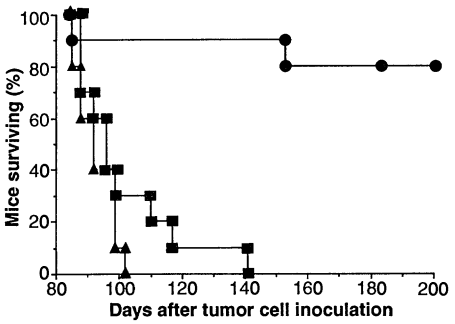

Fig. 3. Conjugates of BR96-DOX cure athymic mice of large disemminated tumors. Mice were inoculated iv with L2987 spheroids and selected approximately 12 weeks later on the basis of visible tumor burden. Selected animals were randomized and left as untreated controls (▲) or treated with BR96-DOX (8 mg/kg) (●) or DOX (8 mg/kg) (■) 82, 86, and 90 days after inoculation of tumor cells.

animals was so far advanced that 50% of control animals died during the first 6 days of the experiment. The MST of the control group was 90 days and 100% of the mice were dead by day 102. Optimal doses of DOX (8 mg/kg) had no effect on the large disseminated tumors; the MST was 94 days and 100% of the mice were dead by day 140. In contrast, mice treated with BR96-DOX (8 mg/kg) had a MST of ≥200 days and eight of the ten animals survived for the duration of the experiment. Surviving mice were killed 200 days after cell inoculation, and sections of the lung, lymph nodes, spleen, colon, jejunum, kidney, liver, brain, and heart were examined by histology. Seven of the eight surviving mice were free of detectable tumor (70% cures by combined life-span and histologic examination).

Although tissues from athymic mice do not bind BR96, normal tissues from several strains of rats, including athymic Rowett rats (Harlan Sprague-Dawley), were shown by immunohistology to bind BR96. The binding of BR96 to rat tissues was similar to that of normal human tissues (*21*), that is, BR96 bound to cells in the esophagus, stomach, and intestine and acinar cells of the pancreas (*15*). We implanted L2987 tumors subcutaneously in athymic rats and initiated therapy when the tumors were 50 to 100 mm^3 in size (Fig. 4). The MTD of unconjugated DOX (4 mg/kg administered as three injections 4 days apart) resulted in a delay in tumor growth and 25% cures. However, BR96-DOX given at a matching DOX dose [DOX (4 mg/kg) and BR96 (140 mg/kg)] cured 100% of the rats, and a dose equivalent to DOX (2 mg/kg) [BR96 (70 mg/kg)] cured 88% of the rats. Of the rats treated with BR96-DOX, 94% (15 out of 16) remained alive and tumor-free with no evidence of toxicity 150 days after the last dose of BR96-DOX.

The BR96-DOX conjugate demonstrated strong antitumor activity in all preclinical models evaluated. The efficacy and potency of BR96-DOX are likely a result of several factors. The antigen to which BR96 binds is abundantly expressed at the tumor cell surface and the active drug is released after antigen-specific binding and internalization of the conjugate into the acidic environment of lysosomes or endosomes. Acid-labile immunoconjugates, in which a less stable disulfide linker was used, have been investigated (*7*, *26*). Although these conjugates were active in an antigen-specific manner, they had poor potency in vivo (*7*). The use of a more stable thioether linker and a mAb with higher avidity and more rapid rates of internalization improved the activity and potency of BR96-DOX and also increased the range of therapeutic doses.

In the studies reported here, administration of BR96-DOX at cumulative doses of at least 15 mg of DOX per kilogram of body weight and 700 mg of mAb per kilogram of body weight [equivalent to DOX (45 mg per square meter of body surface area) and mAb (2100 mg/m^2) (*29*)] resulted in more than 70% cures of established lung tumors. This dose of mAb in mice is approximately equivalent to a cumulative dose in humans of 3 g of mAb per patient and is only slightly higher than that required to achieve saturation of human carcinomas in patients given L6, another anticarcinoma mAb (*30*). The optimal schedule for administering BR96-DOX was not determined in the studies reported here, and the dose of BR96-DOX required to achieve cures may be further reduced with schedule optimization.

The demonstration of tumor cures in animals in which BR96 binds to normal tissues highlights the fact that the appropriate combination of mAb, drug, and linker chemistry are critical aspects to successful antibody-directed therapy. The use of drugs

Table 1. Antitumor activity of BR96-DOX against established human tumor xenografts.

Treatment	Schedule	Injection dose (mg/kg)		Tumor regressions (%)*			Number of mice
		DOX	mAb BR96*	Cures	Complete	Partial	
			Tumor L2987				
BR96-DOX	q4dx3†	20.0	689	100	0	0	8
		15.0	711 ± 36	83.0 ± 0.8	3.3 ± 0.9	7.0 ± 0.9	29
		10.0	513 ± 12	83.0 ± 1.1	8.0 ± 0.7	2.0 ± 0.4	100
		8.0	317 ± 3	88.5 ± 0.1	3.7 ± 1.0	0	27
		5.0	246 ± 5	72.3 ± 2.2	17.9 ± 1.5	5.6 ± 0.7	117
		2.5	109 ± 3	30.4 ± 3.4	33.7 ± 2.4	21.3 ± 2.6	62
		1.25	49 ± 1	6.9 ± 0.9	11.6 ± 1.2	11.9 ± 2.1	44
BR96-DOX	q1dx1‡	30.0	1078	50.0	50.0	0	10
		25.0	930	30.0	30.0	40.0	10
		20.0	735	60.0	20.0	10.0	10
		15.0	540	11.0	22.0	44.0	9
IgG-DOX	q4dx3	10.0	403 ± 5.2	0	0	0	19
		5.0	202 ± 3.2	0	0	3.7 ± 1.0	27
DOX	q4dx3	8.0		0	0	0.8 ± 0.8	125
BR96	q4dx3		400	0	0	0	8
			200	0	0	0	8
			100	0	0	0	8
BR96+DOX	q4dx3	8.0	400	0	0	0	9
		8.0	200	0	0	0	9
		8.0	100	0	0	0	9
			Tumor RCA				
BR96-DOX	q4dx3	20.0	903	100	0	0	10
		15.0	625	80.0	10.0	10.0	10
		10.0	376 ± 5.4	71.7 ± 0.9	0	10.7 ± 0.1	28
		5.0	176	11.0	22.0	11.0	9
		2.5	90	0	0	5.5 ± 1.3	18
BR96-DOX	q7dx3§	20.0	900	100	0	0	10
		15.0	625	100	0	0	10
		10.0	420	80	10	0	10
		5.0	210	10	0	10	10
IgG-DOX	q4dx3	10.0	405	0	0	0	10
DOX	q4dx3	8.0		0	0	0	29
DOX	q7dx3	10.0		0	0	0	10

*Mean ± SEM. †Three injections administered with a 4-day interval. ‡Single injection. §Three injections administered with a 7-day interval.

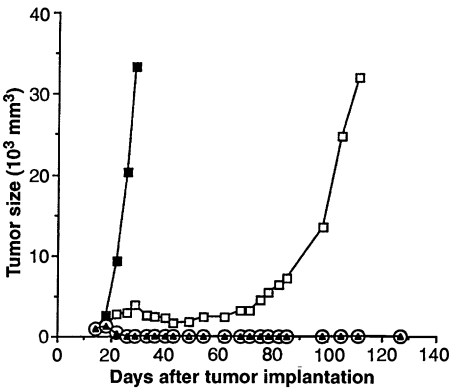

Fig. 4. Conjugates of BR96-DOX cure human lung tumors implanted in athymic rats. Athymic rats that expressed the BR96 antigen in several normal tissues were implanted subcutaneously with L2987 human lung tumors. Therapy was administered 14, 18, and 22 days after the tumor was implanted when tumors were 50 to 100 mm^3 in size. Control animals (■), animals treated with BR96-DOX (4 mg/kg) (○), BR96-DOX (2 mg/kg) (▲), or DOX (4 mg/kg) (□). Data points (○) and (▲) superimpose. The BR96-DOX conjugate at 4 mg/kg cured 100% of the animals, whereas doses of 2 mg/kg cured 88% of the animals and induced complete tumor regression in the other 12%.

such as DOX, with an established clinical profile, may offer a safety advantage over more potent but less defined agents. The toxic effects of DOX are dose-related and it is likely that increasing the intratumoral concentration of DOX will produce a significant increase in antitumor activity (*31*, *32*). Although studies on human tumors growing in immunocompromised animals are not ideal to predict anticancer activity in humans, our findings support the clinical evaluation of BR96-DOX.

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Separable Regulatory Elements Governing *myogenin* Transcription in Mouse Embryogenesis

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Expression of the myogenic helix-loop-helix (HLH) protein myogenin in muscle cell precursors within somites and limb buds is among the earliest events associated with myogenic lineage determination in vertebrates. Mutations in the *myogenin* promoter that abolish binding sites for myogenic HLH proteins or myocyte enhancer factor–2 (MEF-2) suppressed transcription of a linked *lacZ* transgene in subsets of myogenic precursors in mouse embryos. These results suggest that myogenic HLH proteins and MEF-2 participate in separable regulatory circuits leading to *myogenin* transcription and provide evidence for positional regulation of myogenic regulators in the embryo.

The formation of skeletal muscle during development involves a series of events in which multipotential mesodermal stem cells give rise to myoblasts, which ultimately undergo terminal differentiation in response to external cues. Analysis of muscle determination and differentiation in tissue culture has revealed a family of muscle-specific HLH proteins including MyoD, myogenin, Myf-5, and MRF-4, each of which can activate myogenesis in a variety of cell types in vitro (*1*). These myogenic regulators are expressed only in skeletal muscle and are first detected during embryogenesis within myogenic precursor cells in the myotomal compartment of the somites and in the limb buds, localizations consistent with their involvement in myogenic lineage specification (*2–4*).

Activation of muscle-specific transcription by myogenic HLH proteins is mediated by their direct binding to the E box consensus DNA sequence CANNTG in the control regions of most muscle-specific genes (*1*). These regulatory factors also positively auto- and cross-regulate their own transcription in transfected cells (*5*, *6*). Whether autoregulatory interactions among these genes contribute to their expression during embryogenesis or whether this is simply a tissue culture phenomenon is unknown.

An indirect pathway for muscle gene activation has also been described in which myogenin and MyoD induce myocyte enhancer factor–2 (MEF-2) (*7*, *8*), which binds an AT-rich DNA sequence associated with numerous muscle-specific genes (*9*). MEF-2 can activate muscle transcription in the absence of the E box consensus sequence (*10–12*) and is up-regulated when myoblasts enter into the differentiation pathway (*9*). Paradoxically, activation of *myogenin* gene transcription in cultured muscle cells requires binding of MEF-2 to the *myogenin* promoter (*6*). These results suggest that myogenin and MEF-2 participate in a complex regulatory circuit involving positive feedback loops that amplify their expression and stabilize the myogenic program.

Whereas much has been learned about the mechanisms through which myogenic HLH proteins activate muscle-specific transcription in cultured cells, little is known of the mechanisms that regulate muscle gene expression during embryogenesis or of the regulatory circuits that control expression of the myogenic regulators themselves. Because myogenin is the only myogenic HLH protein expressed in all skeletal muscle lines (*13*, *14*), analysis of the mechanisms that control its expression should reveal upstream regulators of myogenic lineage specification. Here, we used *lacZ* transgenes linked to wild-type and mutant *myogenin* promoters to begin to define the regulatory networks that direct *myogenin* transcription in the mouse embryo.

The reporter gene Myo1565lacZ, which contains *lacZ* linked to the region extending from +18 to −1565 base pairs (bp) relative to the *myogenin* transcription initiation site (Fig. 1), was expressed in the same embryonic cells as the endogenous gene (Fig. 2, A and D) and therefore serves as a marker for activation of *myogenin* transcription in individual cells (*4*). Expression of *lacZ* from this transgene can be detected in rostral somites by day 9.0 after coitus (p.c.); expression progresses caudally over the next several days concomitant with somite maturation (*2*, *4*, *13*, *15*). In em-

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